Analysis of Chriz involved in Drosophila polytene chromosome structuring and binding

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Abstract

Drosophila polytene chromosomes are compacted into a series of bands and interbands. Z4 is a protein to keep this pattern of polytene chromosomes, since Z4 mutant larvae show a decompaction of chromosomes and a loss of banding pattern (Eggert et al., 2004). By coimmuno-precipitation, we identified a chromodomain protein, which we named Chriz, for chromodomain protein interacting with Z4 (Gortchakov et al., 2005).

In my PhD thesis, I tested the interactions between the full length proteins and different fragments of Chriz and Z4 which showed that Chriz could directly interact with Z4 in vivo. The interaction domains were mapped and it was determined that the N terminus of Z4 and the C terminus of Chriz are sufficient for mutual interaction. GST pull down confirmed these data and more precisely localized the interaction domains. Chriz, like Z4, is present in many interbands of interphase polytene chromosomes. The overexpression of different domains of Chriz demonstrated that both the N and C terminus are sufficient for targeting of Chriz to interbands. The C terminus was shown to be sufficient for rescue of Chriz null mutations into larva stage. Chriz full length proteins, with site directed mutations within the chromodomain, could still partially rescue the null mutant. Chriz RNAi knock down resulted in a loss of structure of polytene chromosome. The similar chromosomal phenotype of Z4 and Chriz indicate that they cooperate in the formation of chromosomal structure. Using the Chriz RNAi, I showed that Z4 chromosomal binding is dependent on Chriz. However, by a similar assay I showed that Chriz binding did not depend on Z4. Finally, the decondensed interphase chromatin marker Jil-1, a H3S10 histone kinase, and H3pS10 are decreased in Chriz RNAi line.

From these data, I conclude that Chriz/Z4/Jil-1 form an interband binding complex. Chriz is the fundamental factor for the chromosomal targeting and stabilitation of the complex that is required to maintain locally chromatin structure.

Zusammenfassung

Polytäne Chromosomen von Drosophila sind in eine Abfolge von Banden und Interbanden unterschiedlichen Kompaktionsgrades gegliedert. Das Protein Z4 ist notwendig, um dieses Muster aufrecht zu erhalten, da Larven, die für Z4 mutant sind, eine Dekompaktierung von Chromosomen und einen Verlust des Bandenmusters aufweisen (Eggert et al., 2004). Durch Koimmunpräzipitation mit Z4 wurde in unserer Arbeitsgruppe ein Chromodomänen Protein identifiziert, das von uns als Chriz bezeichnet wurde, für: "Chromodomain- Protein interacting with Z4" (Gortchakov et al., 2005).

In meiner Arbeit testete ich die Interaktion zwischen den vollständigen Proteinen Chriz und Z4, sowie verschiedenen Fragmenten beider Proteine. Ich konnte dabei zeigen, dass beide Proteine in vivo direkt miteinander interagieren. Die kartierten Interaktionsdomänen am N-Terminus von Z4 und am C-Terminus von Chriz sind hinreichend für die wechselseitige Interaktion beider Proteine. Die Ergebnisse wurden über GST-Pulldown Experimente abgesichert, wobei die Interaktionsdomänen weiter eingeengt werden konnten. Chriz ist wie Z4 in vielen Interbanden polytäner Interphasechromosomen gebunden. Die Überexpression verschiedener Domänen von Chriz zeigte, dass sowohl der N- als auch der C-Terminus von Chriz für die Interbandenbindung von Chriz ausreichend sind. Der Chriz C-Terminus ist darüber hinaus notwendig, um das Überleben von Tieren mit einer Chriz Null Mutation bis in das larvale Stadium zu gewährleisten. Chriz Proteine mit gezielten Mutationen innerhalb der Chromodomäne konnten ebenfalls Chriz Null Mutationen partiell komplementieren. Tiere mit induziertem Chriz RNAi knock down zeigten eine verringerte DNA Kondensation polytäner Chromosomen. Die Ähnlichkeit des chromosomalen Phänotyps von Z4 und Chriz Mutationen legt nahe, dass beide Proteine in einem gemeinsamen Komplex in Interbanden vorkommen. Unter Ausnutzung von Chriz RNAi bzw. Z4 RNAi konnte ich zeigen, dass die chromosomale Bindung von Z4 von Chriz abhängt. Weiterhin sind die Proteinkinase Jil-1 und an Serin 10 phosphoryliertes H3 (H3pS10), beides Marker für dekondensiertes Chromatin, in Chriz RNAi Tieren verringert.

Aus meinen Daten schliesse ich, dass Chriz/Z4/Jil-1 in einem gemeinsamen Komplex an Interbanden gebunden sind. Chriz ist dabei fundamental wichtig für die zielgerichtete Bindung und Stabilität des Komplexes. Der Komplex selbst ist erforderlich, um die lokale Chromatinstruktur aufrecht zu erthalten.

Contents

Chapter 1

Introduction

The genetic information, present on a linear DNA molecule of considerable length has to be condensed to fit inside a cell nucleus of usually several μm diameter only. Often over one metre of DNA is packaged into the nucleus in an orderly manner that allows for regulation nuclear activities, like transcription, replication and repair, to occur. This is accomplished by the wrapping of DNA around nucleosomes that fold into a structure known as chromatin. This structure is subject to various modifications that have profound influences on gene expression (Schones and Zhao, 2008). Previously, the control of transcription has been considered to be largely dependent on the genetic information provided by local DNA sequences. However, numerous biological phenomena cannot be explained by simple genetics, such as position effect variegation in the Drosophila in which the local chromatin environment of a gene determines its expression or paramutation in korn, that reflects an interaction between two alleles in which one allele causes heritable changes in the other allele (Goldberg et al., 2007). Apparently gene activity depends on the local chromatin structure provided by binding of proteins or RNAs, their modification as well as modification of the DNA molecule itself. Collectively for these processes the name epigenetics was created. Conrad Waddington (1905-1975), embryologist and professor of animal genetics, defined epigenetics as the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being(Waddington,

1942). Epigenetics, in a broad sense, is a bridge between genotype and phenotype, a phenomenon that changes the final outcome of a locus or chromosome without changing the underlying DNA sequence (Goldberg et al., 2007). For example, even though the cells in a multicellular organism share an identical genotype, organismal development produces a diversity of cell types with disparate, yet stable, profiles of gene expression and distinct cellular functions. Cellular differentiation may be considered as epigenetic phenomenon, largely governed by changes in what Waddington described as the epigenetic landscape provided by chromatin modification rather than by alterations in genetic inheritance (Goldberg et al., 2007) (Figure 1.1).

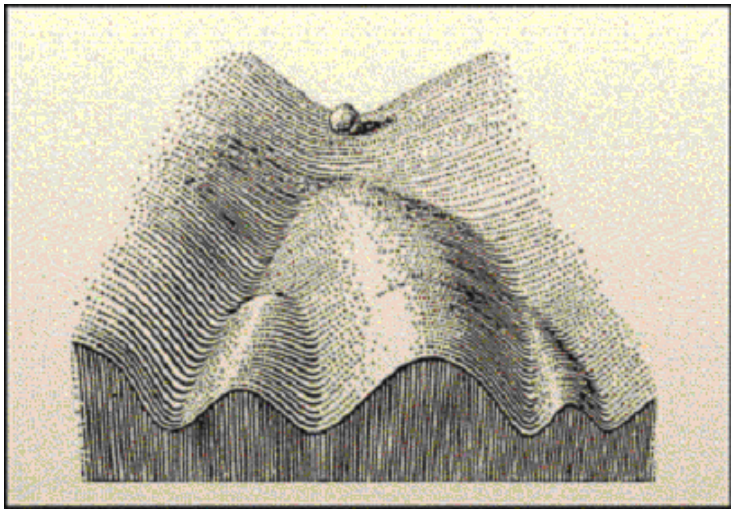

Figure 1.1: Waddingtons Classical Epigenetic Landscape. In 1957, Conrad Waddington proposed the concept of an epigenetic landscape to represent the process of cellular decision-making during development. At various points in this dynamic visual metaphor, the cell (represented by a ball) can take specific permitted trajectories, leading to different outcomes or cell fates (Goldberg et al., 2007).

Today's epigenetic research is mainly focused on the study of covalent modification and noncovalent modifications of DNA and histone proteins and the mechanisms by which such modifications affect chromatin structure and cell fates. Noncovalent means chromatin structural remodelling and the incorporation of histone variants as a way to introduce variation into the

chromatin template. However, since my Phd work will not cover this field I will not introduce this in further detail.

1.1 Chromatin

Chromatin is composed of DNA, RNA and proteins, 50% of which are histones and the remaining proteins collectively called the nonhistone proteins. The basic structural unit of chromatin is the nucleosome. Nucleosomes comprise 147 base pairs of DNA wrapped in a left-handed superhelix 1.7 times around a protein core, the histone octamer. The histone octamer core contains two molecules of each of the histones H2A, H2B, H3 and H4. The DNA connecting adjacent nucleosomes is called linker DNA. In addition to the core histone molecules, the histone H1 is also contained in chromatin and covers part of the linker DNA. DNA in the nucleosome is compacted into the 11nm fiber. This fiber folds into higher order structures, forming chromatin structures that locally differ in their degree of condensation (Figure 1.2).

1.2 DNA Methylation

Locally, the folding of the chromatin is dynamically regulated by several biochemical processes. Two important categories of epigenetic modifications are DNA methylation and histone modifications. DNA methylation is a rather well characterized chemical modification. In mammals, nearly all DNA methylation occurs on cytosine residues of CpG dinucleotides. Regions of the genome that have a high density of CpGs are referred to as CpG islands. Seventy to 80 percent of all CpG sites in human DNA are methylated. DNA methyltransferases are responsible for this modification, which adds activated methyl groups from a donor to cytosine residues at selected sites (Figure 1.3). Mammalian DNMTs have been identified (Bestor TH, 2000). DNMT3A and DNMT3B are thought to be involved in the establishment of DNA methylation while DNMT1 is thought to be involved in the maintenance of this pattern through replication since their substrate is

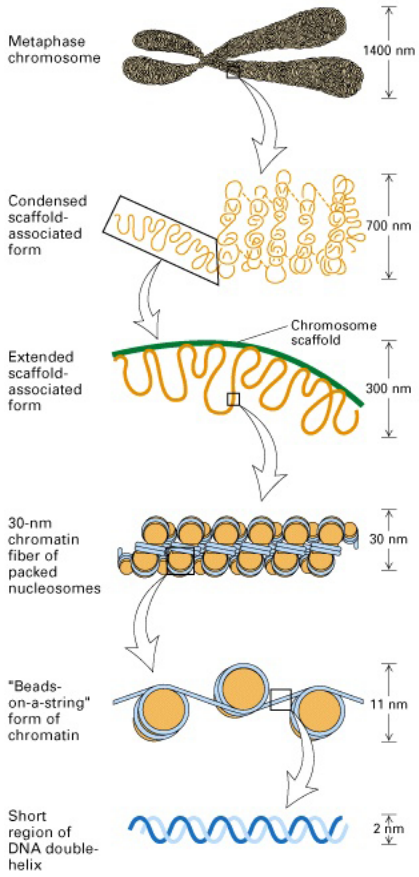

Figure 1.2: DNA double helix coils are around the histone octomer to form a bead-like structure (11 nanometer diameter). histone proteins that form the octamer include H2a, H2b, H3, H4 (2 molecules each) that form a cylinder-like structure that binds DNA. A short sequence of DNA (10-70bp) connects the nucleosomal beads giving it a beads on a string-like appearance. The beads are packed together aided by histone H1 that binds to the beads, pulling them together to form the packed nucleosome or chromatin fiber that has a diameter of 30 nanometers. The chromatin fiber can be further looped or organized onto the nuclear matrix in interphase cells or condensed into chromosomes at metaphase (Rinehart, 2004).

hemimethylated DNA (Okano et al., 1999).

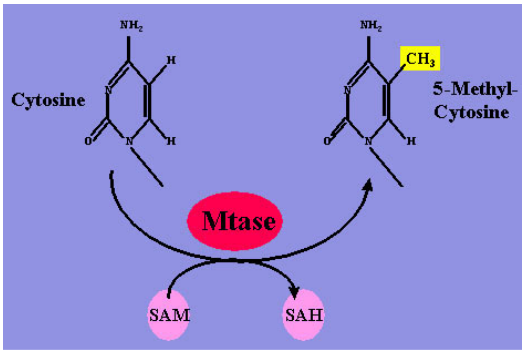

Figure 1.3: DNA methylation refers to the transfer of a methyl (CH3 group) to one of the bases that constitute DNA. The reaction is catalyzed by a DNA methyltransferase (Mtase), and uses S-Adenosyl Methionine (SAM) as a methyl donor. In humans, normal DNA methylation is limited to the Cytosine base (Anderson, 2008).

Alteration of DNA methylation patterns changes gene expression and genome stability by affecting chromatin structure and is therefore associated with a number of disorders (Jones and Baylin, 2002). Mechanistically, a methylated cytosine base can function to promote or preclude recruitment of regulatory proteins. In the former case, the methyl mark can be read through a family of methyl-CpG binding proteins thought to mediate transcriptional repression through interactions with histone deacetylases (Bird, 2002). Alternatively, the methyl mark can exclude DNA binding proteins from their target sites as was shown for CTCF binding at the Igf2/H19 region (Hark et al., 2000). Moreover, the formation of heterochromatin in many organisms is mediated in part by DNA methylation, binding of proteins to this mark in combination with RNA and histone modifications characteristic of silent chromatin (Zaratiegui et al., 2007).

For a long time the fruit fly Drosophila was considered to lack DNA methylation (Rae and Steele, 1979), but now there is evidence for a functional DNA-methylation system in Drosophila as well (Lyko et al., 2000). Drosophila genomic methylation is restricted to the early stages of embryo development. However, the significance of this methylation is unclear until now. DNA methylation in Drosophila later decreases presumably as a result

of reduced methyltransferase expression (Lyko et al., 2000).

DNA methylation plays a role in many cellular processes including silencing of repetitive and centromeric sequences from fungi to mammals, X chromosome inactivation in female mammals and mammalian imprinting, all of which can be stably maintained by the hemimethylated DNA specific DNMT1 (Yang and Kuroda, 2007). Taken together, DNA methylation provides a stable, heritable, and critical component of epigenetic regulation.

1.3 Covalent histone modifications

At the core of the nucleosomes are the highly conserved histone proteins (H3, H4, H2A, H2B). Once thought of as static, structural elements, it is now clear that histones are integral and dynamic components of the machinery responsible for regulating gene transcription. An accumulating data shows post-translational modifications of histone proteins including acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation (Strahl and Allis, 2000) (Figure 1.4). Many of these modifications take place on the 'tail' domains of histones. Indeed, such histone tail modifications can alter DNA-histone interactions within and between nucleosomes and, thus, influence nucleosomal and higher-order chromatin structures (Hansen et al., 1998; Wolffe and Hayes, 1999). Regarding this, histone covalent modifications alone or in combination influence a multitude of cellular processes, including transcription, replication, DNA repair and cell cycle progression.

1.4 Histone Acetylation

Histone acetylation neutralizes the positive charge of the target lysine and occurs at specific lysines on the four core histones (Figure 1.5). As a result, histone acetylation can alter histone-DNA interactions, creating a more open chromatin architecture and it serves as a tag for protein binding (Shahbazian and Grunstein, 2007). This modification is catalyzed by histone acetyltransferases (HATs) through the transfer of the acetyl moiety from

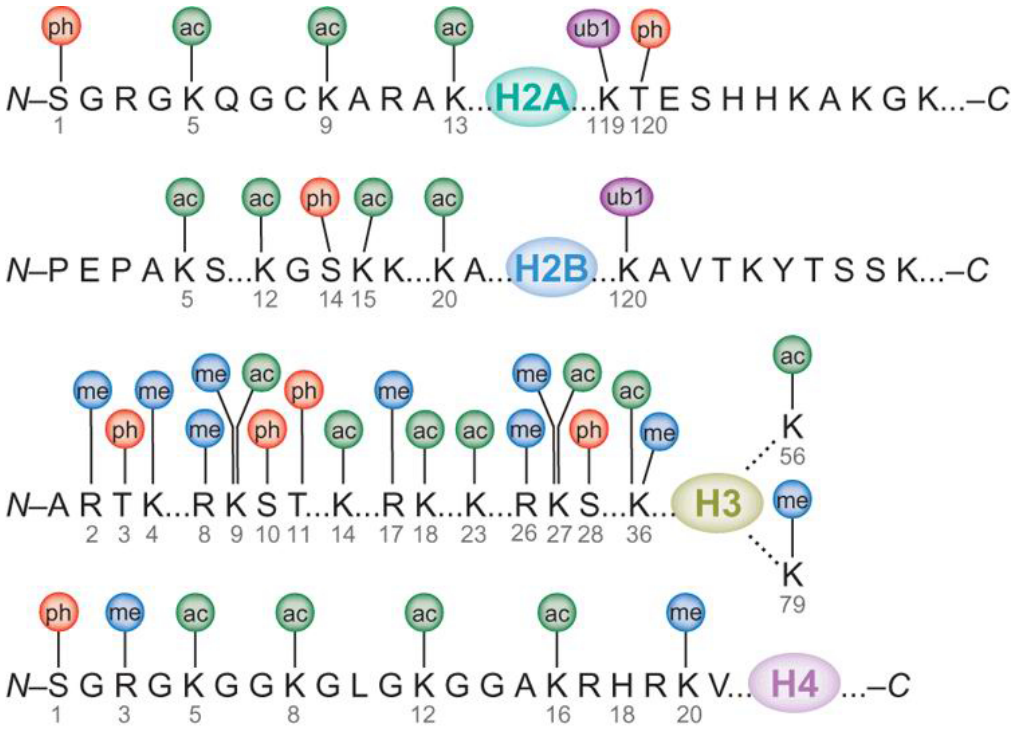

Figure 1.4: The modifications include acetylation (ac), methylation (me), phosphorylation (ph) and ubiquitination (ub1). Most of the known histone modifications occur on the N-terminal tails of histones, with some exceptions including ubiquitination of the C-terminal tails of H2A and H2B and acetylation and methylation of residues within the globular domain of H3 at K56 and K79, respectively. Globular domains of each core histone are represented as colored ovals (Bhaumik et al., 2007).

acetyl-coenzyme A to the ε-amino group of target lysine residues (Table 1.1). Consistently, given that histone acetylation can create a more open chromatin structure, many transcriptional coactivators, such as Gcn5/PCAF, CBP/p300 and SRC-1, have been shown to possess intrinsic HAT activity.

Histone acetylation can be reversed by the enzymatic action of the histone deacetylases (HDACs). The interplay between HAT and HDAC activities thus regulates cellular histone acetylation levels. Complementary to transcriptional coactivators possessing HAT activity, many transcriptional corepressor complexes, such as mSin3a, NCoR/SMRT and NURD/Mi-2, contain subunits with HDAC activity (Denslow and Wade, 2007; Shahbazian and Grunstein, 2007).

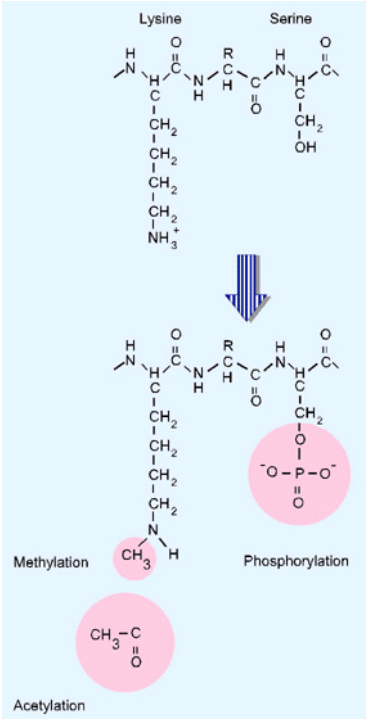

Figure 1.5: Acetylation of lysine or phosphorylation of serine (MBMB, 2007).

1.5 Histone ubiquitination

Histone ubiquitination is catalyzed by the formation of an isopeptide bond between the carboxy-terminal glycine of ubiquitin and the amino-group of a lysine residue on histones. This bond is formed by the sequential catalytic actions of E1-activating and E2-conjugating enzymes and E3-ligases (Shilatifard, 2006; Bhaumik et al., 2007). Whereas the same E1-activating enzyme is involved in the ubiquitination of all target proteins, different E2-conjugating enzymes are required for the ubiquitination of different substrates. E3-ligases provide protein target specificity (Shilatifard, 2006; Bhaumik et al., 2007). Histone ubiquitination can be reversed by deubiquitinases. Often ubiquitination is related to changes in protein conformation or degradation. However, there are also links to gene activation.Ubp8 associates with Gcn5-containing complexes (SAGA and SLIK) and its activity is needed for the full expression of SAGA- and SLIK-regulated genes (Daniel et al., 2004; Bhaumik et al., 2007). An activating role of ubiquitination is reported in transcriptional elongation by the histone chaperone FACT (Denslow and Wade, 2007). The mechanism of ubiquitination for providing this function is unclear. Maybe due to its huge size, ubiquitination physically keep chromatin open through wedging.

1.6 Histone phosphorylation

Histones are phosphorylated at specific sites during cell division (see Figure 1.4) (Barber et al., 2004; Bhaumik et al., 2007). Several distinct kinases are required for the phosphorylation of histones on different residues (Table 1). Phosphorylation of histone H2A is induced by a DNA-damage signaling pathway, and this modification is dependent on phosphatidylinositol-3-OH kinases, such as Mec1 in yeast (Foster and Downs, 2005; Bhaumik et al., 2007). Histone H2B phosphorylation is catalyzed by the sterile-20 kinase in yeast and Mst1 (mammalian sterile-20-like kinase) in mammals (Ahn et al., 2005). Phosphorylation at histone H3S10 and H3S28 during mitosis is regulated by the Aurora kinases, which are highly conserved from yeast to humans

Covalent modifications	Enzymes
H3K4 methylation	Set1 (Sc), SET7/SET9 (Hs), MLL (Hs), Smyd3 (Hs)
H3K9 methylation	SUV39H1 & SUV39H2 (Mm, Hs), G9a (Mm, Hs) Eu-HMTase1 (Hs), ESET & SETDB1 (Mm, Hs), Clr4 (Sp), Dim5 (Nc), Kryptonite (At), Ash1 (Dm)
H3K27 methylation	E(z) (Dm), EZH2 (Hs, Mm)
H3K36 methylation	SETD2/HYPB (Hs), NSD1 (Hs), Set2 (Sc)
H3K79 methylation	DOT1 (Sc), DOT1L (Hs)
H4K20 methylation	Pr-SET7/SET8 (Hs, Dm), SUV4-20 (Hs),SET9 (Sp)
H3R2 methylation	CARM1 (Mm, Hs)
H3R26 methylation	CARM1 (Mm, Hs)
H4R3 methylation	PRMT1 (Hs), RMT1 (Sc)
H3K9 acetylation	Gcn5 (Sc), Src1 (Mm)
H3K14 acetylation	Gcn5 (Tt, Sc), Src1 (Mm), TAF1 (Dm, Hs), CBP & p300 (Hs), Sas3 (Sc),MOZ & MORF (Hs), PCAF & hGcn5 (Hs)
H3K18 acetylation	Gcn5 (Sc), CBP & p300 (Hs)
H3K23 acetylation	Gcn5 (Sc), CBP (Hs), Sas3 (Sc)
H3K36 acetylation	Gcn5 (Sc)
H3K56 acetylation	Rtt109 (Sc)
H4K5 acetylation	Esa1 (Sc), Hat1 (Tt, Dm, Hs), p300 (Hs), Tip60 (Mm), HBO1 (Hs)
H4K8 acetylation	p300 (Hs), Esa1 (Sc), Tip60 (Mm), p300 (Hs), HBO1 (Hs)
H4K12 acetylation	Hat1 (Sc), Esa1 (Sc), Tip60 (Mm) and CBP & p300 (Hs), HBO1 (Hs)
H4K16 acetylation	Mof (Dm), hMof (Hs), Sas2 (Sc), Tip60 (Mm), Esa1 (Sc)
H3S10 phosphorylation	Snf1 (Sc), Jil-1 (Dm), Rsk2 (Mm, Hs), Msk1 (Mm), lp11(Sc), Aurora B (Ce, Dm, Hs), NIMA (An)
H3S28 phosphorylation	Aurora B (Mm, Hs)
H4S1 phosphorylation	Sps1 (Sc), CKII (Sc)
H2BS phosphorylation (S14 in human; S10 in yeast)	Mst1 (Hs), Ste20 (Sc)
H2BK11 acetylation	Gcn5 (Sc)
H2AS129 or H2AXS139 phosphorylation	Tel1 & Mec1 (Sc), ATM & ATR & DNAPK (Hs)
H2AK5 acetylation	Tip60 (Hs, Dm)
H2AK119 ubiquitination	Ring1B (Dm, Mm, Hs)
H2BK ubiquitination (K120 in human, K123 in yeast)	Rad6 (Sc), Bre1 (Sc), HR6A (Hs), HR6B (Hs)
H2AZK14 acetylation	Esa1 (Sc), Gcn5 (Sc)

Table 1.1: The enzymes responsible for covalent histone modifications (Bhaumik et al., 2007). An, Aspergillus nidulans; At, Arabidopsis thaliana; Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; Hs, Homo sapiens; Mm, Mus musculus; Nc, Neurospora crassa; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe; Tt, Tetrahymena thermophila.

(Nowak and Corces, 2004). Other kinases of the MSK/RSK/Jil-1 family can mediate phosphorylation of histone H3 at Ser10 during interphase that is correlated to gene activity (Nowak and Corces, 2004; Bhaumik et al., 2007).

In *Drosophila*, H3S10 phosphorylation is also found in dividing cells (See (Adams et al., 2001)) and the gene responsible for phosphorylation of H3S10 at mitosis is known as Aurora B. In Aurora B RNAi cells, the phosphorylation of H3S10 decreased during mitosis and resulted in a failure to recruit condensin to the chromosomes (Adams et al., 2001; Giet and Glover, 2001). As a consequence many defects were observed including lack of sister kinetochore separation, lagging chromatids, and extensive chromatin bridging at anaphase. These data support that the phosphorylation of histone H3S10 is essential for the regulation of mitotic processes. However, phosphorylation of H3S10 is also observed during interphase. The transcriptionally active heat-shock locus usually shows significant H3S10 phosphorylation. Intriguingly, Labrador (Labrador and Corces, 2003) observed that when transcription was driven from an hsp70 promoter, H3S10 was hyperphosphorylated, but when transcription was driven from a P-element transposase promoter, phosphorylated H3S10 was not detected. Therefore, histone H3S10 phosphorylation may be only required in the context of specific promoters. Phosphorylation of the histone H3S10 residue was also described to be associated with chromosomal condensation (Hendzel et al., 1997; Wei et al., 1999). However, as already mentioned phosphorylation of H3S10 also has been found to occur at transcriptionally activated heat shock puffs (Nowak and Corces, 2000) implying a role for phosphorylation of histone H3S10 in establishment of the decondensed state in addition to its role in chromosome condensation (Mizzen et al., 1998; Thomson et al., 1999). Furthermore, it has been demonstrated that MSK1/2 kinase activity and histone H3S10 phosphorylation have roles in chromatin remodeling and gene transcription in mammals (Dunn et al., 2005). Thus, histone H3S10 phosphorylation is associated with two opposed chromatin states, highly condensed mitotic chromosomes and the relaxed chromatin of activated genes during interphase (Prigent and Dimitrov, 2003). This apparent contradiction has sparked the speculation that the effect of the H3S10 modification on chromatin structure might be

context dependent and be influenced by adjacent epigenetic marks such as histone acetylation or H3S28 phosphorylation (Turner, 2000; Johansen and Johansen, 2006).

1.7 Histone methylation

Histones may be methylated on lysine residues, arginine residues or both. Histone arginine methylation can occur in the mono- or dimethylated form at specific positions on histone H3 and histone H4 in higher eukaryotes (Figure 1.4). Histones also can be mono-, di-, or trimethylated on lysines 4, 9, 27, 36 and 79 of histone H3 and Lys20 of histone H4. The machinery and the sites of histone methylation are highly conserved from yeast to humans, in particular for methylation of H3K9, H3K27 and H4K20 (Kouzarides, 2007). Unlike HATs, which can be promiscuous in their histone substrate specificity and can modify several residues within the same or different histones, histone methyltransferases are typically more specific for their targets. Almost all of the histone lysine methyltransferases characterized to date contain a SET domain, named after D. melanogaster Su(var)3-9, Enhancer of zeste (E(z)), and Trithorax (Trx) enzymes. SET domain containing enzymes can catalyze methylation of specific lysines on histones H3 and H4 (Table 1). Although some histone methyltransferases share the same substrate specificity, it is likely that each enzyme may regulate different genes or different cellular processes in vivo.

Set1 and Set2 have been shown to methylate Lys4 and Lys36 of histone H3, respectively (Shilatifard, 2006). Histone H3K4 methylation is a hallmark of actively transcribed genes. Therefore, the identification of complexes that mediate this modification has been the focus of many laboratories (Shilatifard, 2006). The yeast Set1 protein associates with seven other polypeptides to form the COMPASS (complex of proteins associated with Set1). Several COMPASS components contain WD domains, also found in other trithorax-related complexes (Shilatifard, 2006). COMPASS was the first identified H3K4 methyltransferase and can catalyze the mono-, di- and trimethylation of this residue (Shilatifard, 2006; Bhaumik et al., 2007).

More recently it was found that histone lysine methylation is also reversible. Demethylation is carried out by two families of enzymes, amine oxidases such as LSD1 and hydroxylases of the JmjC family (Schneider and Shilatifard, 2006; Klose and Zhang, 2007; Shi, 2007). Because LSD1 requires a protonated methyl ammonium group, only mono- and dimethyl forms can be substrates. In contrast, JmjC family members can also demethylate trimethylated lysines (Klose and Zhang, 2007; Shi, 2007). LSD1 can demethylate Lys4 or Lys9 of H3, depending on its associated proteins (Klose and Zhang, 2007; Shi, 2007). Many JmjC family members have unique substrate specificities, with demethylases for H3K4, H3K9, H3K27 and H3K36 being characterized recently (Shi, 2007). Despite the theoretical ability of JmjC members to demethylate mono-, di- or trimethylated lysine, it seems that some JmjC family members are more specialized. For example, D. melanogaster Lid only demethylates H3K4me3 (trimethylated H3K4) to H3K4me2 in vivo, although its mammalian homologs can also convert H3K4me2 to H3K4me1 (Bhaumik et al., 2007; Eissenberg et al., 2007; Iwase et al., 2007).

Histone methylation plays different roles in biological processes depending on the site and type of the histone modified. Arginine methylation of histones H3 and H4 regulates transcriptional activation of steroid-responsive genes (Lee et al., 2005). Histone lysine methylation is also involved in the transcriptional process since Lys4 methylation on histone H3 in present at the 5 end of transcription units and is associated with the early elongating form of RNA polymerase II at actively transcribed genes (Shilatifard, 2006). H3K36 methylation by Set2 is associated with the elongating form of RNA polymerase II and is found more towards the 3 end of transcribed genes (Shilatifard, 2006). In contrast, H3K27, H4K20 and histone H3K9 methylation is linked to heterochromatin formation. The Suv39 protein methylates histone H3K9 and localizes to transcriptionally silent heterochromatin, where it recruits the transcriptional repressor protein HP1 (Richards and Elgin, 2002; Ayyanathan et al., 2003; Grewal and Moazed, 2003). HP1 in turn recruits more Suv39 and both recruit H4K20 histonemethyltransferases which place further silence marks. However, the molecular mechanism of chromatin com-

paction by this protein complex is not known in detail. Like histone H3K9 methylation, histone H3K27 methylation is involved in gene silencing (Shilatifard, 2006; Sims and Reinberg, 2006; Bhaumik et al., 2007). This often alters developmentally regulated genes that have to be permanently silenced in certain tissues.

1.8 Chromatin organization and histone modifications

Histone modifications main functions are: the establishment of global chromatin environments and the local targeting of proteins or protein complexes for the orchestration of DNA-based biological processing. As to establish a global chromatin environment, modifications help to partition the genome into distinct domains such as euchromatin, where DNA is kept accessible, and heterochromatin, where chromatin is inaccessible for transcription. This may be a very local function, such as the modification of a nucleosome at the promoter or it may be more global like in the regulation of chromosomal domains or whole chromosomes. In any case, this requires the ordered recruitment of the machinery to unravel DNA, manipulate it and keep it an altered state or eventually to reset it to the initial chromatin state.

Text books describe two different types of chromatin environments in the genome, silent heterochromatin and active euchromatin. In reality however, both eu- and heterochromatin may be further subdivided in domains different in structure and function. Each of these domains is associated with a distinct set of modifications.

Heterochromatin that makes up to 30% of the total genome has a low gene density, a compacted appearance revealed by intense staining and is late replicating in S-phase. Heterochromatin is required for the protection of chromosome ends and functional centromeric heterochromatin is important for the separation of chromosomes in mitosis. Formation of facultative heterochromatin is required for X inactivation and silencing of developmental regulators to avoid inappropriate expression. In mammals the silent hete-

rochromatic state is associated with low levels of acetylation and high levels of methylation of H3K9, H3K27 and H4K20. The recruitment of PRC-1 to H3K27me is required for developmental gene silencing and thought to be involved in the maintenance of the inactive X chromosome. The recruitment of HP1 to H3K9me is thought to play an important role in the maintenance of pericentric heterochromatin silencing.

Euchromatin is a large proportion of the genome. In this environment DNA has extended flexibility for biological output. Genes can be turned on or kept off, DNA can be unravelled for repair or replication. Euchromatin is less condensed reflected by faint staining of chromatin. It has a higher gene density and replicates early in S-phase. The histone modification pattern in euchromatin reflects this open chromatin state. In the transcriptionally inactive state, already significant levels of acetylation, methylation, and phosphorylation can be detected on genes, but these are still insufficient to elicit transcription. Further enzymatic activities are necessary for transcription to take place and typically, actively transcribed euchromatin has high levels of acetylation and is trimethylated at H3K4, H3K36, and H3K79.

Impressive evidence for switching of opposing chromatin states (heterochromatin and euchromatin) emerged from (Ebert et al., 2004). Three copies of the Su(var)3-9 H3K9 methyltransferase result in a strong enhancer of PEV phenotype. This strain also shows increased H3K9 di and trimethylation throughout the genome, notably, also in many euchromatic regions. Conversely, mutants were isolated showing a strong suppressors of PEV phenotype. These mutants were special alleles of the Su(var)3-1 gene, which encodes the Jil-1 kinase that controls phosphorylation of H3S10 within euchromatin (Wang et al., 2001) and all alleles contained mutations in the C terminus of Jil-1, leaving the kinase activity unchanged. These striking results show that Su(var)3-1 can completely antagonize the function of Su(var)3-9 in heterochromatin assembly and spreading, and that it may act to maintain a balance between euchromatin and heterochromatin. This antagonistic relationship is in support of the histone switch hypothesis (Fischle et al., 2003a) in which the functional readout of local chromatin may be modified by opposing histone modifications, such as methylation and phosphorylation.

Opposing chromatin modifications also can be responsible for creation of insulator elements required as boundaries between euchromatin and heterochromatin that are established or regulated by mutual exclusion or inhibition of different histone modifications as shown for the globin domain (West et al., 2004). The insulator elements can be classified as enhancer-blocking or barrier elements depending on whether they interfere with enhancer-promoter interactions or act as barriers against the spreading of heterochromatin. The latter class functions as described: in the globin domain the spreading of H3K9 trimethylated heterochromatin is blocked by USF dependent establishment of a local patch of H3K9 acetylated chromatin (West et al., 2004). The former class may exert its function by attaching the chromatin fiber to a nuclear substrate such as the nuclear matrix, resulting in the formation of structural or functional chromatin loops. The best known examples of such looped domains are the globin loci in vertebrates, in particular the chicken β-globin locus. In chicken red blood cells the globin genes are coordinately regulated within a 33 kb chromosomal domain, which differs by its chromatin structure from the flanking chromatin already early in development. Inherent to the concept gene activities within a given domain are kept insulated from the activity of adjacent domains. The globin domain is flanked by two constitutive hypersensitive regions, HS4 and 3'HS, whose function as boundary elements was shown by their ability to act as enhancer blockers and by conferring position-independent expression of transgenes in vertebrates and invertebrates. In addition, the CTCF protein, which also was identified as an essential boundary element factor in other species, is needed for their function. Upstream, the HS4 element blocks the spreading of a 16 kb heterochromatic chromatin region and inhibits the regulatory crosstalk with the strong folate receptor gene enhancer. The downstream element keeps the expression of the globin genes independent from that of the immediate flanking odorant receptor gene, which is normally expressed in olfactory epithelia and certain neurons only (Bell et al., 2001; Bulger et al., 2002; Eggert et al., 2004).

In *Drosophila*, the heat-shock locus in 87A7 is a well studied domain model. The two divergently transcribed hsp70 genes are flanked by strong

DNAseI hypersensitive sites which are part of the so called specialized chromatin structure elements scs and scs', respectively (Udvardy et al., 1985). On heat-shock induction these elements mark the edges of a decondensed puff formed at this site. Both scs and scs' elements function as boundary elements. They confer position-independent expression on Drosophila transgenes and are functional as enhancer blockers in transgene expression (Kellum and Schedl, 1991). The insulating activity of the scs element is mediated by the Zeste-white 5 protein (Zw5) (Gaszner et al., 1999), whereas the scs' activity is mediated by the boundary element associated factor 32 (BEAF-32) (Zhao et al., 1995). It was shown that Zw5 and BEAF-32 proteins interact. Results from ChIP experiments using Drosophila cell lines provided evidence that the scs and scs' elements are close in space to each other in nuclei in vivo, although they are separated by 15 kb of intervening sequence on linear DNA (Blanton et al., 2003). This is consistent with a role of these elements in forming the base of a looped domain.

1.8.1 Drosophila polytene chromosome

Polytene chromosomes are a good model to study chromatin organisation since they have reproducible band-interband pattern that is visible in normal light microscopy. By cytophotometry and high resolution in situ hybridization data, the DNA packaging ratio in bands was determined as 60 to 100 fold and that of interbands to about 5 to 8 fold (Rykowski et al., 1988). Thus, interband chromatin structure is likely a 10-nm nucleosome fiber, whereas chromatin in bands is condensed at least into 30nm fibers (Rykowski et al., 1988).

Polytene chromsomes are interphase chromosomes common to many Diptera, plants and Ciliates. Through repeated rounds of DNA replication without mitosis and cell division (called endoreplication), they become large bundles of chromatids with their chromomeres in register. As a consequence of polytenization, interphase chromosome organization becomes apparent where phase dark bands are separated by phase light interbands, resulting in a species-specific banding pattern. (see Figure 1.6). For unknown reasons,

the centromeric regions of the chromosomes are less replicated. As a result, the centromeres of all the chromosomes bundle together in a mass called the chromocenter.

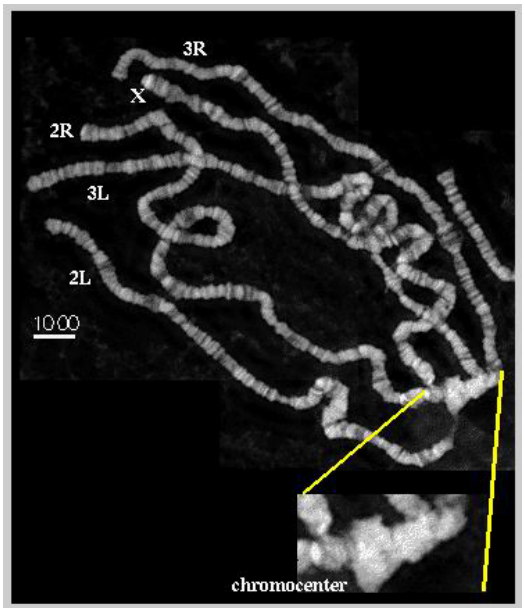

Figure 1.6: Drosophila polytene chromosomes are viewed on light microscopy. Chromosome arms and chromocenter are labelled (SEDAT, 2008).

Polytene chromosomes are usually found in the larvae, reflecting the mode of growth at that stage that occurs by hypertrophy of cells mainly. Because each cell now has many copies of each gene, it can express its genes more efficiently than with only two copies as in diploid cells. A similar pattern of bands is consistently observed in all polytene tissues down to the lowest level of polyteny amenable to cytological analysis. Thus, the band/interband pattern may reflect a common structural organization of interphase chromosomes in general. Intuitively, the difference in compaction between bands and interbands suggests the existence of different mechanisms of formation of the two elements. This difference in chromatin organization may reflect important aspects of how gene expression is regulated (Semeshin et al., 2008).

1.9 Previous work of my project

However, little is known about the molecules and molecular mechanisms that are responsible for controlling the establishment and maintenance of these chromosomal subdivisions. In order to address this question interband specific binding proteins are investigated in our group. We cloned the Z4 gene which encodes an 996 amino acid protein with a calculated molecular mass of 113KDa that is found in about 80% of all interbands. The protein has 7 zinc fingers of the classical C2H2-type, between amino acid 239-515 (Eggert et al., 2004). Z4 is an essential protein that is ubiquitously present in all tissues during embryonic and larval development and exerts its function within interphase, as it does not bind to the chromosomes during mitosis. Z4 null (Z4-1.3) and hypomorphic (Z4-7.1) mutants were obtained by imprecise excision. These mutations can be complemented by the 7.4 kb genomic region encoding the complete Z4 gene, resulting in viable adults. The homozygous hypomophic mutation, Z4-7.1 shows a progressive disintegration of the chromosomal structure. Regions of the polytene chromosomes loose their band/interband organization and develop a decompacted and cloudy appearance. Z4 can directly bind to DNA, however so far no sequence specific interaction has been found. It is still unclear, how Z4 is involved in structuring the chromosome. A possibility may be the modification of nucleosomes. More recently a Z4 mutation was found independently that shows growth defects and therefore was called Putzig (Kugler and Nagel, 2007). As was shown in their paper, a consequence of down regulation of Z4 in the loss of H3K4 methylation in imaginal disc nuclei. H3K4-trimethylation is also a hallmark of interband chromatin. However, it still has to be demonstrated how loss of H3K4 methylation is connected to loss of Z4.

The Chriz protein was first found by coimmunoprecipitation with Z4-specific antibodies. Mass spectrometric analysis of gel purified protein identified a chromodomain protein with an apparent molecular weight of about 140 kDa which was named Chriz, for Chromodomain protein interacting with Z4 (Eggert et al., 2004). Chriz is a single copy gene located in 79F on chromosome 3L close to the centromeric heterochromatin (Gortchakov

et al., 2005). The chromodomain, an evolutionary conserved protein module of about 50 amino acids, is located at the N terminal part of Chriz. Heterochromatin protein 1(HP1) and Polycomb (PC) also have chromodomains which mediate the specific recognition of methylated lysine residues K9 and K27 of histone H3, respectively (Nielsen et al., 2002; Fischle et al., 2003b). Comparison of the Chriz chromodomain sequence with those of HP1 and PC reveals significant conservation in residues. Nevertheless, two out of the three aromatic residues in the chromodomain of HP1/PC that were identified to be crucial for the recognition of histone H3 di- and trimethylysine are substituted in the chromodomain of Chriz for charged amino acids. Besides the chromodomain, no other strongly conserved domains were found in the protein database. Chriz perfectly colocalizes with Z4 and like Z4, Chriz is absent from bands and from decondensed puffs. The latter observation rules out the possibility that selective binding to interbands would simply be explained by enhanced accessibility. Chriz is essentially required for development and also dosage sensitive since the ubiquitous overexpression of Chriz under the control of act-Gal4 and T80-Gal4 driver lines is early larval lethal. Using different driver lines with a more restricted GAL4 expression pattern, organ- and tissue- specific defects were observed. By imprecise excision, Chriz null and hypomorphic mutants were obtained which can be complemented with a 7.1kb genomic Chriz fragment (Gortchakov et al., 2005).

Chriz was independently identified as Chromator, as an interaction partner of the putative spindle matrix component Skeletor, that localizes to the spindle and to the centrosomes during mitosis (Rath et al., 2004). Furthermore, functional assays using RNAi-mediated depletion in S2 cells suggest that Chromator directly affects spindle function and chromosome segregation (Rath et al., 2004). However, localization of Chromator to polytene interbands suggested them that it also has a functional role in maintaining chromatin structure during interphase. They performed an EMS mutagenesis screen that generated two new Chro hypomorphic alleles. The analysis of these alleles showed that impaired Chromator function leads to disorganization and misalignment of band/interband regions resulting in coiling and folding of the polytene chromosomes (Rath et al., 2006). In addition, they

demonstrated that Chromator directly interacts with JIL-1 kinase and that the two proteins extensively co-localize at polytene interband regions(see below). These findings suggest that Chromator and JIL-1 interact in an interband-specific complex to establish or maintain polytene chromosome structure in Drosophila.

Drosophila Jil-1 is a tandem kinase, which localizes specifically to euchromatic interband regions of polytene chromosomes. Jil-1 is upregulated almost 2-fold on the hypertranscribed male X chromosome compared to autosomes. (Jin et al., 1999) and is the predominant kinase regulating histone H3S10 phosphorylation at interphase (Wang et al., 2001). Analysis of Jil-1 null and hypomorphic alleles showed that Jil-1 is essential for viability and that reduced levels of JIL-1 protein lead to a misalignment of interband polytene chromatin fibrils that is further associated with coiling of the chromosomes and an increase of ectopic contacts between non-homologous regions (Zhang et al., 2003; Deng et al., 2005). This results in a shortening and folding of the chromosomes with a non-orderly intermixing of euchromatin and the compacted chromatin characteristic of banded regions (Deng et al., 2005). The intermingling of non-homologous regions can be so extensive that these regions become fused and confluent, further shortening the chromosome arms. Based on these findings a model was proposed where JIL-1 functions to establish or maintain the parallel alignment of interband chromosome fibrils as well as to repress the formation of contacts and intermingling of non-homologous chromatid regions (Deng et al., 2005). Reducing the level of JIL-1 results in the spreading of the major heterochromatin markers dimethyl H3K9 and HP1 to ectopic locations on the chromosome arms, with the most pronounced increase on the X chromosomes. Genetic interaction demonstrated that JIL-1 functions in a pathway that includes Su(var)3-9 (Zhang et al., 2006). By using a LacI-tethering, it was shown that Jil-1 mediated ectopic histone H3S10 phosphorylation that is sufficient to induce a change in higher-order chromatin structure from a condensed state to a more open euchromatic state(Deng et al., 2008).

1.10 The aim of my work

Studies of the relationships between nuclear architecture and chromatin binding proteins and histone modifications reveal novel principles underlying nuclear compartmentalization (Branco and Pombo, 2007). The chromatin associated complex Z4/Chriz/Jil-1 was identified by us and others and accumulating evidence shows that it may play a role in the maintenance of the Drosophila polytene chromosome structure. The proteins colocolize in most interbands, they interact with each other and mutants result in loss of chromatin structure. However, there are still many questions to be answered. So the aim of my work is to address the mechanism of the complex function in Drosophila chromatin structure. How do the proteins interact with each other? Z4 and Chriz were identified by our group by CoIp. It is clear that they are in the same complex, but whether they interact directly is not known. Both of the proteins have conserved domains, and I became interested if these domains may mediate the observed interactions. The second question was how the proteins and the complex as a whole were targeted to interbands. Z4, Chriz and Jil-1 all are interband specific proteins whereby Z4 and Chriz have identical chromosomal distribution, colocalizing largely with Jil-1. Having identified the targeting region it would be interesting to unravel which protein is the fundamental factor for interband targeting of the complex. The third question relates to the function of the complex in interbands. Interbands are considered as less condensed compared to bands. Intuitively, this is related to local chromatin modification. So I will use available RNAi lines to knockdown the Chriz and Z4 protein level to elucidate consequences on protein binding, modification and chromatin structure.

Chapter 2

Material and Methods

2.1 General used molecular biological applications

2.1.1 Bacteria and Yeast strains

The E.coli strains, XL-1, BL21 and the yeast strain SFY56 used for protein expression and for protein-protein interaction testes are summarized in Table 2.1.

Strains	Genotype	Reportergene
E. coli XLI	*recA1 endA, gyrA96 thi-1 hsdR17 supE44 relA1, lac [F', proAB, lacIqZDM15, Tn10 (Tetr)]c*	
E. coli BL21 (DE3) pLysS	*E. coli B, F-, dcm, ompT, hsdS($r_B^- m_B^-$), gall(DE3) [pLysS Camr].*	
S. cerevisiae SFY526	*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, can^r, gal4-542, gal80-538,URA3::GAL1$_{UAS}$-*GAL1$_{TATA}$-LacZ	LacZ

Table 2.1: Bacteria and yeast strains used in this thesis.

The E.coli strain XL-1 was used for the cloning and amplification of plasmids. The BL-21 was used for the expression of protein. The yeast

strain SFY was used for the protein-protein interaction in yeast two hybrid assay.

2.1.2 Plasmids

All plasmids that have been used in this work are listed in Table 2.2. The property of these plasmids, their application, resistence and their source supply are as indicated. All of these plasmids have the ability to be replicated in E. coli cells and they allow the selection of cells which contain them since they give their host bacteria an antibiotic resistance. The plasmids pGEX-2T, pGEX-6p-1 and pET $-$ $(\text{Myc})_3$ $-$ $(\text{His})_6$ are used to express GST- and MH- recombinant proteins respectively. The pUAST are P-element based plasmids used to incorporate genes into the Drosophila genome by embryonic injection. pπ25.7 wc plasmids are used to express transposase to help pUAST integrate into genome of fruit fly.

Additionally, two other yeast plasmids, pGBT9 and pGAD424 were used in the yeast two hybrid system for analysing protein-protein interaction. The selection marker for these two plasmids is the growth of the yeast cells of on medium lacking Tryptophan/Leucin (Trp/Leu). The pGAD424 and pGBT9 plasmids were applied to express Activation Domain (AD) fusion proteins and DNA Binding Domain (DBD) fusion proteins respectively.

2.1.3 PCR and cloning of plasmid constructs

Different plasmids which were used for the working with yeast, bacteria and fruit fly were constructed. The cloning of these plasmids occurred in the following procedures.

Polymerase chain reaction (PCR)

Polymerase chain reaction provide a basis to amplify DNA fragments and to create also a new enzyme restriction site in the amplified DNA fragment needed for the cloning. Specific enzyme restriction sites were introduced by

Name	Application	Resistence/ selection marker	Source of supply
PET3a-Myc-His	Myc-His fusion protein expression in BL21 E.coli cell	Amp/-	Novagen Rosenberg et al., 1987;
PGEX-6p-1	GST fusion protein expression in BL21 E.coli cells	Amp/-	Invitrogen
PGEX-2TK	GST fusion protein expression in BL21 E.coli cells	Amp/-	Pharmacia Biotech Smith and Johnson, 1988
pUAST	Expression in Drosophila (Gal4/UAS system)	Amp/ mini white gene	Brand & Perirrimon 1993
Pπ25.7 wc	Helper Plasmid	Amp/-	Lab stock
pGBT9	DBD fusion protein expression in yeast cells	Amp/ TRP	Clontech
pGAD424	AD fusion protein expression in yeast cells	Amp/LEU	Clontech

Table 2.2: Lists of plasmids are used for cloning strategies and for expression of fusion proteins. Shown are also the application, the resistance and selection marker and the source of supply of each of these plasmids.

using the following primers (Table 2.3) in order to amplify and clone the Chriz and Z4 constructs.

PCR is performed using the following standard reaction mixture:

Template DNA	50-100 ng
10x amplification buffer	1:10 of the final volume
Mixture of dNTPs	4 μM
Primer1	10 μM ($\simeq$100pmol)
Primer2	10 μM ($\simeq$100pmol)
Taq DNA polymerase	5 Units
H_2O to a final volume of 50μl	

Primer used in this work

Name	Primer sequence	Enzyme site
chrizR72	Ttagcgtcgactcgatcctaatggctatgc	SalI
chrizR92	Ttagcgtcgactacgttgggatgttgagcg	SalI
chrizF92	Tctagtcgacggcacgccaaaaacttact	SalI
chrizR82	Ttagcgtcgacttggtcttggaagttcgag	SalI
chrizF82	Tctagtcgacatcaccaaccggtaagctg	SalI
chrizF72	Tctagtcgacaatgttggcacaggagattt	SalI
chrizF73	Gtattgcggccgcttggcacaggagatttc	NotI
chrizF83	Gtattgcggccgcaccagaaaatcaccaac	NotI
chrizF93	Gtattgcggccgcgcacgccaaaaacttac	NotI
chrizF4g	Gtcagatctttggcacaggagatttcacct	BglII
chrizF5g	Gtcagatctcgcatagccattaggatcgat	BglII
chrizF6g	Gtcagatctcaattggcacgccaaaaact	BglII
chrizR7	Ttagcggtacctcgatcctaatggctatgc	KpnI
chrizR8	Ttagcggtaccttggtcttggaagttcgag	KpnI
chrizR9	Ttagcggtacctacgttgggatgttgagcg	KpnI
chrizF4	tctggatccttggcacaggagatttcacct	BamHI
chrizF5	tctggatcccgcatagccattaggatcgat	BamHI
chrizF6	tctggatcccaattggcacgccaaaaact	BamHI
chrizR4	Gacgaattctcgatcctaatggctatgcg	EcoRI

chrizR5	Gacgaattctccagacccttctccctagt	EcoRI
chrizR6	Gacgaattcttacgttgggatgttgagcg	EcoRI
chrizF7	Atcgaggtacctaatgttggcacaggagat	KpnI
chrizF8	Atcgaggtaccaatcaccaaccggtaagct	KpnI
chrizF9	Atcgaggtacctggcacgccaaaaacttac	KpnI
chrizF10	Gcggccgctgcagcagtcgctaagcgcttc	NotI
chrizR10	Tgctgcagcggccgcctcttcggtgcgctg	NotI
chrizF11	Gatgaagagaccgacgttgatcgctcgcat	
chrizR11	Gtcggtctcttcatcctcgtgggtcttacg	
chrizF12	Gccgcgttcgatgacgtgatggccaatctc	
chrizR12	Gtcatcgaacgcggcatgatgcgagcgatc	
chrizF1	Tacgaattcttggcacaggagatttcacct	EcoRI
chrizF2	Tacgaattccgcatagccattaggatcgat	EcoRI
chrizF3	Tacgaattccaattggcacgccaaaaact	EcoRI
chrizR1	Tgactgcagtcgatcctaatggctatgcg	PstI
chrizR2	Tgactgcagtccagacccttctccctagt	PstI
chrizR3	Tgactgcagttacgttgggatgttgagcg	PstI
chrizF10-1	Gccgcagtcgatgacgtcgctaagcgcttc	
chrizR10-1	Gtcatcgactgcggcctcttcggtgcgctg	
Z4R1	Tgacagatctcttggtggccttgcgttgga	BglII
chrizF2-1	Tgacgaattctcctcagttccctctgccgg	EcoRI
Z4F1	Tgacgaattcaacaaccaactgaatccggc	EcoRI
Z4R2	Tgacagatctagttgccttcttgtcacgtc	BglII
Z4F2	Tgacgaattcgtccaacgcaaggccaccaa	EcoRI
Z4F3	Tgacgaattcagacgtgacaagaaggcaac	EcoRI
Z4R3	Tgacagatctgtcggtctttgtctccgtaa	BglII
Z4R4	Tgacctcgagcttggtggccttgcgttgga	XhoI
Z4R5	Tgacctcgagagttgccttcttgtcacgtc	XhoI
Z4R6	Tgacctcgaggtcggtctttgtctccgtaa	XhoI
CF1	Gactgaattccttactagggagaagggtct	EcoRI
CR1	Gactctgcagcgaaacgggtgccggtgcct	PstI

CF2	Gactgaattccgggttgaaaattccgaggc	EcoRI
CR2	Gactctgcagtacctggtaaacagtgccgt	PstI
CF3	Gactgaattccaagctcagttgtgtccaat	EcoRI

Table 2.3: List of the primers used in this work. F: forward primer; R: reverse primer.

Overlap PCR

Overlap Extension PCR is used to create long DNA fragments from short ones or changing its genetic code through mutations. In this work, I used overlap PCR to create Chriz chromodomain mutant1 and mutant3, in which the mutant amino acids were introduced by PCR primers. First Chriz primer F73 and R10-1, F10-1 and R9 were used as two pair primers to amplify two fragments using Chriz gene as template. These two fragments have 15 bp overlap, in which were the mutated sequence. Gel extraction was used to clear up the 690bp and 2100bp correct size band. The new round PCR reaction was set up using cleaned up fragments as "template" without adding new primers into the reaction tube. Proofreading enzyme was used for extension. Ten PCR cycles were run without end primers (Template extension step). F73 and R9 primers were added, then continue PCR cycling for another 20 rounds. The corrected band Chriz mutant 1 (2770bp) product was extracted from Gel. Chriz mutant1 contruct were cloned into pUASTmychis vector with NotI and KpnI enzyme. Chriz mutant 3 were also constructed in this way by using F73-R11, F11-R9, F73-R12 and F12-R9 primers. See Figure 2.1 for procedure of overlap PCR.

2.1.4 Digestion of DNA with restriction enzyme

Enzymes were purchased from New England Biolabs. For each digestion, the following solution were added and mixed in a 1.5 ml microcentrifuge tube.

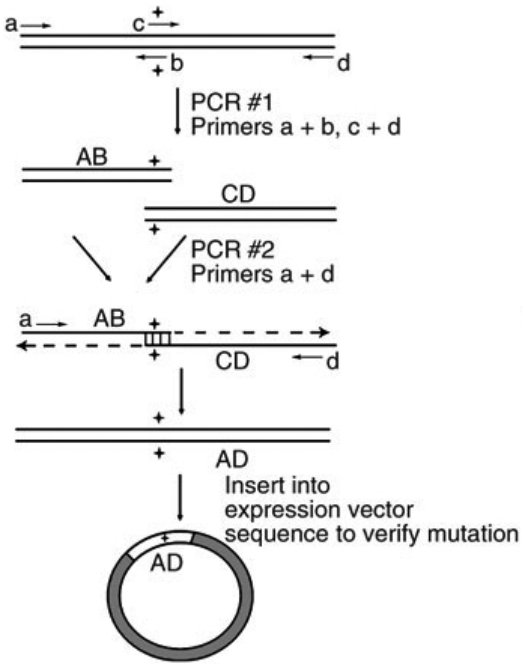

Figure 2.1: Procedure of overlap PCR (Heckman and Pease, 2007).

DNA	up to 1 μg
10×buffer	2 μl
Enzyme	10 unit
H_2O	up to 20 μl

The mixture was incubated at 37°C for 2-3 hours.

2.1.5 DNA ligation

The ligation of the insert (PCR fragment) to the specific plasmids (vectors) was prepared as following.

In a microcentrifuge tube the following reaction mixture were mixed and incubated for 16 hours at 22°C (Table 2.4).

2.1.6 Setting up competent cells

The preparation of competent bacteria was done according to Hanahan protocol (Hanahan, 1983) with changing.

Linear vector DNA	5-10 μl (50-400 ng)
Insert DNA	use a 1:1 up to a 3:1 molar ratio of insert DNA to vector DNA
10× ligation buffer for T4 DNA Ligase	2 μl
Water	to 20 μl
T4 DNA Ligase	0.2-0.4 μl (1-2 u) for sticky ends; 1μl (5 u) for blunt ends

Table 2.4: DNA digestion procedure.

First day, frozen stock of XL1 bacteria were streaked on LB plate and incubated overnight at 37°C. The second day, a single colony was inoculated into a 4ml LB medium, which grow overnight with shaking at 37°C. The next day, one flask containing 400 ml LB medium were set up. Two ml overnight culture was given to the flask. The cell culture was incubated with shaking until it reaches an OD600 of $\sim$ 0.4-0.5. Bacteria are centrifuged at 4000 rpm at 4°C for 10 minutes and resuspend the pellet on ice gently in 60 ml TFBI. After cooling on ice for 10 min, centrifuge bacteria at 4000 rpm, 4 °C for 10 minutes. Resuspend the pellet gently on ice in 8 ml of TFBII and immediately dispense 100 μl of cell suspension into each cold microcentrifuge tubes on ice. Once all 8 ml of competent cells are dispensed into microcentrifuge tubes, immediately freeze them in liquid nitrogen and stored at -80°C.

- LB medium:
  - Tryptone (10 g/l)
  - Yeast extract (5 g/l)
  - NaCl (5 g/l)
- TBFI solution:
  - 100 mM Rubidium Chloride
  - 50 mM Manganese Chloride
  - 30 mM Potassium Acetate

  - 10 mM Calcium Chloride
  - %15 w/v Glycerol
  - Adjust pH to 5.8 with acetic acid 2M and Sterilize by filtration

- TFBII solution:
  - 10 mM MOPS
  - 10 mM Rubidium Chloride
  - 75 mM Calcium Chloride
  - 15% w/v Glycerol
  - Sterilize by filtration

2.1.7 Bacterial Transformation

Frozen competent cells were taken out from -80°C and place on ice, waiting for cells to thaw. Note: Keep cells chilled on ice to ensure high transformation efficiency.

Before adding DNA, it is better to mix cells by flicking the tube gently, then 100 μl per transformation was taken into a sterile pre-chilled (on ice) microcentrifuge tube. 1-50ng of DNA (in a volume no greater than 10μl) was added to 100μl cells. Quickly flick the tube several times to ensure the even distribution of DNA. Tubes were immediately placed on ice for at least 10 minutes. Heat shock the cells for 45-50 seconds in a water bath at exactly 42°C without shaking. Then tubes were immediately placed on ice for 2 minutes. 800μ of room temp (or 37°C) LB medium were added and incubated for 1 hour at 37°C with shaking at ∼150 rpm. 100-800 μl of the transformation mix was plated onto antibiotic plates. The plating volume depends on the concentration of DNA. The cells may be pelleted by centrifugation at 1000 rpm for 1 minute, then the cells can be resuspended in 50-200 μl of LB medium and plated. (The maximum amount of solution that may be spread on a plate is ∼200 μl). For the positive control DNA, a 1:100 to 1:1000 dilution is recommended for plating on LB plates. Plates were put in the 37°C incubator and grow overnight 14-18 hrs depending on

the cell growth rate (XL1 usually grow slower than BL21; it's better to keep an eye on the growth of the cells the next day).

For the positive control DNA, a 1:100 to 1:1000 dilution is recommended for plating on LB plates. Plates were put in the 37°C incubator and grow overnight 14-18 hrs depending on the cell growth rate (XL1 usually grow slower than BL21; it's better to keep an eye on the growth of the cells the next day).

2.1.8 Mini and Midi DNA preparation

Mini DNA preparation was done according to standard molecular cloning protocol. You may find the detail in molecular cloning book (Sambrook et al., 2001). The midi DNA preparation was done according to Hispeed plasmid midi kit protocol (Qiagen).

In this work, I have constructed 35 plasmids for bacterial expression, yeast two hybrid and fly work respectively. All the plasmids are listed as following. Their names, cloning enzyme sites and protein regions are also indicated (Table 2.5).

Name of plasmids	Used primers	Regions (AA)	Cloning site
pGAD424chriz	chrizF1R3	2-926	EcoR1, PstI
pGAD424chrizN	chrizF1R1	2-211	EcoR1, PstI
pGAD424chrizM	chrizF2-1R2	193-285	EcoR1, PstI
pGAD424chrizC	chrizF31R3	273-926	EcoR1, PstI
pGAD424chrizN M	chrizF1R2	2-285	EcoR1, PstI
pGAD424chrizMC	chrizF2-1R3	193-926	EcoR1, PstI
pGBT9Z4	Z4F10R10	1-996	EcoRI, BglII
pGBT9Z4N	Z4F1R1	1-237	EcoRI, BglII
pGBT9Z4M	Z4F2R2	231-522	EcoRI, BglII
pGBT9Z4C	Z4F3R3	516-996	EcoRI, BglII
pGAD424chrizC1	chrizCF1CR1	279-509	EcoRI, PstI
pGAD424chrizC2	chrizCF2CR2	500-768	EcoRI, PstI
pGAD424chrizC3	chrizCF3CR3	700-926	EcoRI, PstI

pGEX2TKchrizN	chrizF4R4	2-211	BamHI, EcoR1
pGEX2TKchrizM	chrizF5R5	193-285	BamHI, EcoR1
pGEX2TKchrizC	chrizF6gR6	273-926	BglII, EcoRI
pGEX2TKchrizNM	chrizF4R5	2-285	BamHI, EcoRI
pGEX2TKchrizMC	chrizF5gR6	193-926	BglII, EcoRI
pGEX2TKchriz	chrizF4gR6	2-926	BglII, EcoRI
pGEX-6p-1chrizN	chrizF1R72	2-211	EcoRI, SalI
pGEX-6p-1chrizM	chrizF2-1R82	193-285	EcoRI, SalI
pGEX-6p-1Z4N	Z4F1R4	1-237	EcoRI, XhoI
pGEX-6p-1Z4M	Z4F2R5	231-522	EcoRI, XhoI
pGEX-6p-1Z4C	Z4F3R6	516-996	EcoRI, XhoI
pET28achrizNM	chrizF1R82	2-285	EcoRI, SalI
pET28achrizC	chrizF31R92	273-926	EcoRI, SalI
pUASTMycHischrizN	chrizF73R7	2-211	NotI, KpnI
pUASTMycHischrizCD	chrizF83R8	160-327	NotI, KpnI
pUASTMycHischrizC	chrizF93R9	275-926	NotI, KpnI
pUASTMycHischrizNCD	chrizF73R8	2-327	NotI, KpnI
pUASTMycHischrizCDC	chrizF83R9	160-926	NotI, KpnI
pUASTMycHischrizM1	chrizF10-1R10-1	2-926	-
pUASTMycHischrizM3	chrizF12R12,F11R11	2-926	-
pGAD424chrizC1+2	chrizCF1CR2	279-768	EcoRI, PstI
pGAD424chrizC2+3	chrizCF2CR3	500-926	EcoRI, PstI

Table 2.5: list of constructs in this work.

In addition to the above plasmids, the following plasmids were also used in this work. They were constructed by former Phd students Gortchakov AA(Gorchakov et al., 2004). Table 2.6.

Name of plasmids	Regions(AA)
pMHchriz	29-926
pMHchrizΔHindIII	29-710
pMHchirzΔSmaI/pstI	29-590
pMHchrizΔSacI PstI	29-456
pMHchrizΔBamHI/PstI	29-346
pMHchrizΔHindIII	29-291

Table 2.6: List of constructs taken from Gortchakov AA.

2.2 Protein -protein interaction assays

2.2.1 Yeast two hybrid assay

In order to study Chriz-Z4 interaction, I performed yeast two hybrid assay and GST pull down. Yeast two hybrid system is working with SFY526 yeast cells. Two plasmids pGBT9 and pGAD424 are used to achieve Chriz-Z4 interaction test. The truncations of Chriz gene were cloned into pGBT9 and pGAD424, the truncations of Z4 gene were were cloned into pGBT9 as shown in Table 5. Yeast competent cells and LiAc yeast transformation procedure are as following.

One ml of YPD medium was inoculated with several colonies, 2-3 mm in diameter from the fresh prepared working stock plate. Vortex vigorously for 5 min to disperse clumps and were transferred into a flask containing 50 ml of YPD. Incubate at 30°C for 16-18 hr with shaking at 250 rpm to stationary phase (OD_{600} 1.5). Next day, transfer 30 ml of overnight culture to a flask containing 300 ml of YPD. The OD600 of the diluted culture were measured up to 0.2-0.3. Incubate at 30°C for 3 hr with shaking at 230 rpm until an OD600 of 0.4-0.6. Cells were placed in 50 ml tubes and centrifuge for 5 min at 1000 rpm at room temperature.

The supernatants were discarded and thoroughly resuspend the cell pellets in 25-50 ml sterile TE or distilled H_2O. Centrifuge at 1000 rpm for 5 min at room temperature. The supernatants were discarded. The cell pellets were resuspended in 1.5 ml of freshly prepared, sterile 1X TE/1X LiAc. Add 0.1 μg pGBT9Z4, 0.1 μg pGAD424Chriz plasmids DNA and 0.1 mg herring

testes carrier DNA to a fresh 1.5 ml tube and mix. Since I wanted to test different fragments of Chriz and Z4 interaction, so different combination of pGBT9Z4 fragments with pGAD424Chriz fragments were added to individual tubes respectively. 0.1 ml of yeast competent cells were added to each tube and mixed well by vortexing. 0.6 ml of sterile PEG/LiAc solution was added to each tube and vortexed at a high speed for 10 sec to mix. Incubate all the tubes at 30°C for 30 min with shaking at 200 rpm. Later added 70 μl of DMSO to each tube and mixed well by gentle inversion. In a 42°C water bath heat shocked them for 15 min. Chill cells for 2 min on ice. Cells were centrifuged for 5 sec 14,000 rpm at room temperature. Then I removed the supernatant. Cells were resuspended in 0.5 ml of sterile 1XTE buffer. Finally, the cells were plated on SD agar plates lacking Trp and Leu nutrient that will select for the desired transformants.

Plates were incubated at 30°C for 2-4 days until colonies appear. The growing clones indicated that both pGBT9Z4 and pGAD424Chriz plasmids were successfully transformed into the yeast cells.

- YPD medium:
  - 20 g/l Difco peptone
  - 10 g/l yeast extract
  - 20 g/l agar (for plate)
- PEG/LiAc solution (polyethylene glycol/lithium acetate) per 10 ml solution:
  - PEG 4000: 8 ml of 50% PEG
  - TE buffer: 1ml of 10X TE
  - LiAc: 1ml of 10X LiAc
- 50% PEG:
  - 50g PEG4000 in 100 ml sterile deionized H_2O
- 10× TE buffer:

  - 0.1M Tris-HCl, 10 mM EDTA, PH 7.5. Autoclaved

- 10× LiAc:

  - 1 M lithium acetate, pH 7.5 Autoclaved

β galactosidase assays

In order to test the interaction between Chriz and Z4, β galactosidase assays was performed. I used the colony-lift filter assay and liquid culture assay to qualitively and quantitively measure the interaction. The colony lift filter assay and liquid culture assay were shown as following.

The colony-lift filter assay For each plate (for example, the colonies in this plate contained both pGBT9Z4 and pGAD424Chriz plasmids) to be assayed, a sterile whatman filter was presoaked by placing it in 2.5-5 ml of Z buffer/X-gal solution in a clean plate. Using a forceps, a sterile dry whatman filter was placed over the surface of the plate of colonies to be assayed. Gently the filter was rubed with the side of the forceps to help the colonies cling to the filter. The filter was oriented to the agar by poking holes through the filter into the agar. Carefully lifted the filter off the plate and transfered it, colonies facing up, to a pool of liquid nitrogen and submerged completely the filters for 10 sec then removed it from the liquid nitrogen and allowed it to thaw at room temperature. Finally, carefully placed the filter, colony side up, on the pre-soaked filter and incubated it at 30°C or at room temperature until the appearance of blue colonies. Meanwhile, the positive control should be done. Once there was no blue appearance, one can easier find out the reason. The blue colour should appearance within 3-4 hours, more than this, it may be a mask. One should be careful with this point. By this assay, I can tell which two fragments interacted, but I can't conclude which interaction is stronger. In order to answer this question, liquid culture assay was performed.

Liquid culture assay using ONPG as substrate This assay is used to verify and quantify two protein interactions. ChrizC, ChrizCDC and

ChrizC1+2 interacted with Z4. Also, ChrizC, ChrizCDC, ChrizC2, ChrizC1-+2 and ChrizC2+3 interacted with Chriz. In order to quantify these interactions, the following procedure was done.

A positive colony which containing pCL1 plasmid and the above described colonies were picked from the original plate to a 5 ml fresh SD selection medium and incubated at 30°C overnight. On the day of the experiment, ONPG was dissolved at 4 mg/ml in Z buffer with shacking for 1-2 hours. Two ml of the overnight culture from different colonies were transfered to 8 ml of YPD respectively and incubated them for 3-5 hr at 30°C with shacking 250 rpm until the cells were in mid log phase with OD600 of 0.5-0.8. The exact OD600 was recorded when harvesting the cells. One and a half ml of culture from different colonies were placed into each of three microcentrifuge tubes respectively and centrifuged at 14,000 rpm for 30 sec. Carefully removed supernatants and added 1.5 ml of Z buffer to each tube and vortexed until cells were resuspended. Cells were centrifuged and supernatants were removed. Pellets were resuspended in 300 μl Z buffer (the concentration factor is 1.5/0.3= 5 fold) and 0.1 ml of the cell suspension were transfered to fresh tubes. I placed them in liquid nitrogen until the cells were frozen, then placed the tubes in a 37°C water bath to thaw. The freeze/thaw cycles were repeated two more times to ensure that the cells have broken. A control blank tube was setted up with 100 μl of Z buffer. 0.7 ml of Z buffer + β-mercaptoethanol was added to the reaction and blank tubes. After I added160 μl of ONPG in Z buffer at 30°C, started to record the time. After the yellow colour developed, 0.4 ml of 1 M Na_2CO_3 was added to the reaction and blank tubes. The elapsed times from different tubes were recorded in minutes.

Reaction tubes were centrifuged for 10 min at 14,000 rpm and supernatant were transfered to clean cuvettes and the OD_{420} of the samples relative to the blank tube were measured.

The β-galactosidase units were calculated in the following way:

- β-galactosidase units = 1,000 $\times OD_{420}/(t \times V \times OD_{600})$, where
  - t=elapsed time (in min) of incubation

  - V=0.1 ml × concentration factor
  - OD_{600} = A_{600} of 1 ml of culture

  One unit of β-galactosidase is defined as the amount, which hydrolyzes 1 μmol of ONPG to o-nitrophenol and D-galactose per min per cell. Data are shown in results part of this work.

- Z buffer:
  - 16.1 g/L $Na_2HPO_4 \cdot 7H_2O$
  - 5.50 g/L $NaH_2PO_4 \cdot H_2O$
  - 0.75 g/L KCl
  - 0.246 g/L $MgSO_4$ adjust pH 7.0 and autoclave
- X-gal:
  - dissolve X-gal in DMF at a concentration of 20 mg/ml. It was stored at -20°C in the dark.
- Z buffer/ X-gal:
  - 100 mL Z buffer
  - 0.27 mL β-mercaptoethanol
  - 1.67 mL X-gal stock solution

2.2.2 GST pull down assay

The pull-down assay is an *in vitro* method used to determine physical interaction between two proteins. Pull-down assays are used for confirming the existence of a protein-protein interaction predicted by other research techniques (e.g., co-immunoprecipitation, yeast two-hybrid). I have tested the interaction between Chriz and Z4 in yeast two hybrid, further I wanted to use pull down assay to confirm these interactions. Since the His-tagged Chriz truncations were available, so Z4 N terminus was cloned to a GST tagged vector pGEX-6p-1. The procedure of GST pull down assay is indicated as following.

Expression of fusion proteins

pMHchriz (aa29-926), pMHchrizΔHindIII (aa29-710), pMHchrizΔSphI HindIII (aa29-291), pMHchrizΔSacI PstI (aa29-456), pMHchrizΔBamHI/PstI (aa29-346), pMHchirzΔSmaI/pstI (29-590) constructs were cloned by former Phd students Gortchakov AA.

After conforming these plasmids, they were transformed into the *E-coli BL21* cells and incubated over night at 37°C after plated on the amp antibiotic plates. Then a single colony from the empty pGEX-6p-1 vector and from each recombinant plasmids pGEX-6p-1-Z4N, pMHchrizΔSphIHindIII (aa29-291), pMHchrizΔSacIPstI (aa29-456), pMHchrizΔBamHI/PstI (aa29-346), pMHchirzΔSmaI/pstI (aa29-590) were inoculated 2 ml of LB medium and incubated overnight at 37°C with shaking. Next day, the cultures were diluted 1:100 into fresh LB medium and grown at 37°C with shaking until the OD_600 reached a value between 0.6 - 0.8.

IPTG (to a final concentration of 0.1 mM) were added the cultures to induce the protein expression and incubated at 37°C for another 90 min with shaking. The cultures were centrifuged at 4000 × g for 10 min at 4°C. After the pellets were suspended in ice-cold PBS (50 μl PBS per ml cell cultures), the cells were lysed by adding of 20mg/ml lysozyme to a final concentration of 1mg/ml for 20 min. The bacteria cells were sonicated on ice in short burst 3 times for 16 S at 50 amplitude (Bandelin sonoplus HD70). The probes were centrifuged at 12,000xg for 10 min at 4°C. After the analysis of the presence of respectively fusion proteins on Coomasie staining, the supernatants, cells extracts, were transferred to a fresh tube and stored at -20°C.

- PBS:
  - 7 mM Na_2HPO_4
  - 3 mM NaH_2PO_4
  - 130 mM NaCl
  - pH 7.4

Invitro protein interaction test using GST beads

The prepared cell extracts of Z4N GST were purified with GST beads (detail see GST purification protocol from Amershan Bioscience). Ten μl purified GST-proteins (according to the amount of protein, about 2 μg) bound to the slurry sepharose beads were taken into 5 microcentrifuge tubes seperatively and mixed with 200 μl pull down buffer. 4 μl cells extracts from different Myc His fusion proteins were added into 5 microcentrifuge tubes. They were incubated at 4°C for 2 hours with continuous shaking. As a control the GST alone beads was incubated in the same work condition with Myc-His different fusion proteins respectively. Then the probes were centrifuged for 3 min at 4°C 2000rpm. The supernatants were discards. The beads were washed with washing buffer 5 times, each time washing rotate 10min at 4°C. Finally 20 μl SDS loading buffer was added and cooked for 5 min at 95°C then analysed by SDS-PAGE.

- Pull down buffer:
  - 20mM Tris pH 7.5
  - 0.1mM EDTA
  - 150mM NaCl
- Washing buffer:
  - 20mM Tris pH 7.5
  - 0.1mM EDTA
  - 150mM NaCl
  - 0.3% Triton

When I performed proteins interaction assays, SDS-PAGE, Coomassie staining and Westen Blot techniques were used. The procedure of above methods was described in Molecular Cloning book (Sambrook et al., 2001). I will not discuss these methods in detail.

2.3 Antibodies

The anti-Jil rabbit antiserum was the generous gift of G.Reuter. The rabbit anti-Chriz polyclonal antibody and mouse anti-Z4, anti-myc monoclonal antibody was described in Eggert et al (Eggert et al., 2004). The anti-lamina antibody was described (Errede and Ammerer, 1989). The anti-GST, anti-H3 and anti-H3pS10 antibodies were obtained from commercial sources (Sigma and Abcam, respectively). The secondary antibody of the polytene chromosome squashing was a Rhodamine or FITZ conjugated goat anti-mouse or goat anti-rabbit antibody (Dianova).

2.4 Fly work

2.4.1 Flies strains

The ued fly stains in this work are as in Table 2.7. All these flies are stocks from H. Saumweber's lab. Other P element inserted fly lines were used in this work are listed in results part.

2.4.2 Fly food

- 8.5g agar
- 10g soja flour
- 18g dry yeast
- 81.6g maltextract
- 40.8g dextrose
- 76.6g corn flour

They are mixed in 800 ml water under heating. Then 0.6g Nipagin and 4.5 ml propionic acid were added and mixed again. Then the media were poured into the bottles. After cooling down, these bottles were added fresh yeast paste and maintained at 4 degree for 2 week.

Fly name	Marker	Application	Chromosome
WT-Oregon	Red eyes	As control	
W^{1118}	White eyes	Germline transformation	
Tft/Cyo	curly wing and tufted bristles	Balancer line	2nd
TM3/TM6	Stubble larvae and short bristles	Balancer line	3nd
Gal4 231.1	Red eyes	Gland driver line	2nd
Gal4 SGS58	Red eyes	Gland driver line	2nd
Engrailed Gal4	Red eyes	Wing disc posterior region driver line	2nd
T80 Gal4	Red eyes	Ubiquitious driver line	2nd
ChrizRNAi	Orange eyes	Knock down Chriz	X
Z4RNAi	Red eyes	Knock down Z4	3rd
ΔKG3/TM6		Chriz null mutant	3rd

Table 2.7: Flies strains.

2.4.3 Fruit juice medium

40g agar was dissolve in 1000 ml water under heating. 16.6g sugar and 3.6g nipagin was dissolved in 300 ml fruit juice at 70°C. Then the fruit juice mixed with agar solution. 100 ml glacial acetic acid was added. After mixing and cooling down, the media were poured into small plates. They were stored at 4 degree. Before used, the fresh yeast paste was added.

2.4.4 Microinjection of Drosophila embryos

Transgenic flies were obtained by microinjection of the gene-carried pUAST plasmids into the preblastoderm embryos with the helper plasmid Pπ25.7 wc. Microinjection requires a couple of technical devices like a micropippet puller to produce the injection capillaries, a micromanipulator, a microinjector, and a microscope. Whereas the microscope is needed to optically control the microinjection procedure, the micromanipulator helps to gently align the injection capillary with the embryos. Finally, the microinjector is used to control the delivery of a reproducible amount of injection mixture to the embryos (Bachmann and Knust, 2008). The detail one can find in fly book. This injection work was done with the help of R. Gienapp and D. Zhao.

2.4.5 Crossing map of rescue assay

Since Chriz null mutant is available, I would like to know which part of Chriz is essential for its normal function, so I performed rescue assay. Gal4T80/Cyo line was used as driver line. ΔKG3/TM6 was used as null mutant line. The homozygous of ΔKG3 line is embryonic lethal. P[Chriz] means P element insertion of Chriz different fragments.

After two generation crossings, in F2 generation I obtained the flies with desired genotypes. P[chriz] over Cyo and ΔKG3 over TM6. Gal4-T80 driver line over Cyo and ΔKG3 over TM6. The F2 generation flies were identified with different eye color. $\frac{Gal4-T80}{Cyo}i\frac{\Delta KG3}{TM6}$ has dark red eyes. $\frac{p[chriz]}{Cyo}i\frac{\Delta KG3}{TM6}$ has orange eyes. The F3 generation has 4 potential genotypes for each transgenic P[chirz] construct:

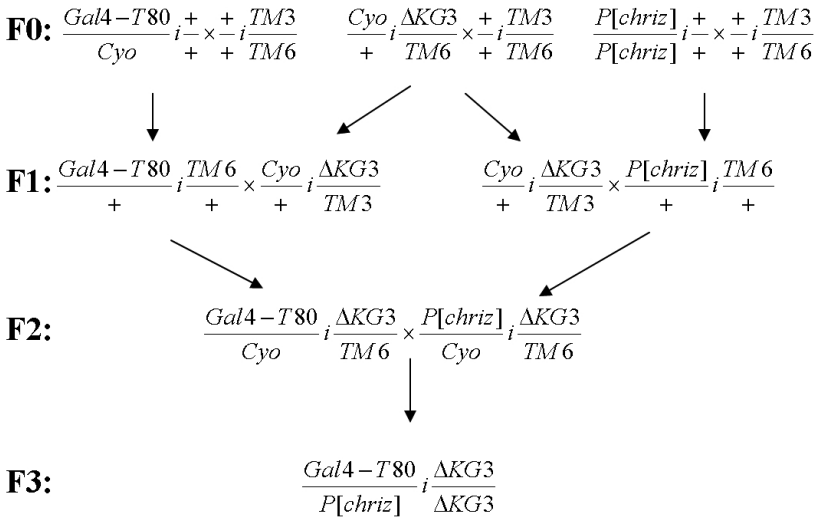

Figure 2.2: Crossing map of rescue assay.

$\frac{GalT80}{Cyo} i \frac{\Delta KG3}{TM6}$, $\frac{GalT80}{P} - i \frac{\Delta KG3}{TM6}$, $\frac{P}{Cyo} i \frac{\Delta KG3}{TM6}$, $\frac{GalT80}{P} i \frac{\Delta KG3}{\Delta KG3}$ each genotype can be identified by different phenotype. $\frac{GalT80}{Cyo} i \frac{\Delta KG3}{TM6}$ dark red eye, Cyo, TM6 $\frac{GalT80}{P} i \frac{\Delta KG3}{TM6}$ dark red eye, non-Cyo, TM6, $\frac{P}{Cyo} i \frac{\Delta KG3}{TM6}$ orange eye, Cyo, TM6 $\frac{GalT80}{P} i \frac{\Delta KG3}{\Delta KG3}$ dark red eye, non-Cyo, non-TM6. The results are described in next chapter.

2.4.6 Preparation of imaginal discs

Imaginal discs were dissected from third instars larvae in PBS and collected in cold PBS. The dissected material was placed in fix solution for 20-30 min at room temperature. After fixation, the discs were washed with PBT three times for 10 min each. Then they were put in block solution for 30min at room temperature. Then they are ready for immunostaining.

- Fix solution: 4 % formaldehyde in 1× PBS
- PBT: 0.3 % Triton X-100 in 1× PBS
- Block solution: 5% FCS in 1× PBS

2.4.7 Immunostaining of imaginal discs

The probes weere incubated overnight at 4°C with the primary antibody. They were washed three times with 1× PBT. After that, they were incubated for 2 hour with the Rhodamin-marked secondary antibody. The imaginal discs were washed three time with 1× PBT and stained with Dapin for 20 min. Using a pipette, a drop of 87% glycerol was placed onto a 22×22 mm slide and the imaginal discs were diped to the drop then the coverslide was put onto it. The excess of the glycerol was cleaned up. The slide was viewed immediately or stored at -20°C in the dark.

2.4.8 Squash preparations of polytene chromosomes

The glands were dissected from 3rd instar larvae in a drop of prep solution on a slide. They were transfered for 30 sec into a drop of fix solution and briefely into a drop of squasch solution on the same slide. Then each lobe was transfered separately to 12.5μl of squasch solution on an 18×18mm coverslip. The gland was left on coverslip for 3-4min while preparing another pair of glands. The coverslip was taken up using a fresh slide. The glands were sheared by sliding the coverslip upside the slide in all directions, tapered with blunt forceps (to the edges of coverslip only) to shear them again and squash them by blotting away excess liquid. The quality of squash was inspected at 10×phase microscope. If it is good enough, then it was freezed by plunging it into liquid nitrogen. Coverslip was taken off with a razor blade from one of its edges. The slide was marked the field of squash and the upside of it with a diamond pen. The good squashes were collected in 96% alcohol for at least 10min (longer times do not hurt). Then they were washed twice 10min in buffer A and incubated for 3h with first antibody in a moist chamber at room temperature (alternatively at 4°C overnight). They were washed twice 10min in buffer A again. Then they were incubated for 1-2 h with second antibody in a moist chamber at room temperature. They were washed twice 10min in buffer A by adding 0.2 μg/ml Hoechst dye to the second washing. Then again two times washing were done in buffer A. A drop of 87% glycerol was placed onto a 22×22 mm coverslip using a pipette. The slide was diped

with the area containing the chromosomes to the drop. The excess of the glycerol was cleaned up. The slide was viewed immediately or stored at -20°C in the dark.

- Buffer A 10×stock solution:
  - 150mM Tris HCL, PH 7.4
  - 600mM KCL
  - 150mM Nacl,
  - 5mMSpermidine,
  - 1.5mM Spermine
- Prep solution:
  - 100μL 10% Triton×100
  - 100μL 10×buffer A
  - 800μL H_2O
- Fix solution:
  - 100μL 10% Triton×100
  - 100μL 10×buffer A
  - 100μL 37% formaldehyde
  - 700μL H_2O
- Squash solution:
  - 100μL 37% formaldehyde
  - 400μL H_2O
  - 500μL glacial aceic acid

2.4.9 Preparation of gland extracts from third instar larvae

In order to analysis the protein level in salivary gland, protein extract from gland were prepared. Glands were dissected from larvae and put into a microcentrifuge tube with cold PBS. When the expected number of glands was collected, then SDS loading buffer was added. They were immediately loaded into SDS gel after heating at 95°C 5min or stored in -20°C.

Chapter 3

Results

3.1 Molecular interaction between Chriz and Z4

3.1.1 Chriz directly interact with Z4 in yeast

On Chromosomes Chriz and Z4 are present in a common complex since Chriz could be co-immunoprecipitated from Kc cell extracts with Z4 antibodies. Immunostaining of polytene chromosome also shows that Chriz and Z4 are exactly colocalized. However, it was not clear if there was a direct interaction between both proteins and if so, which protein domains were required for such an interaction. So in order to obtain evidence for direct interaction, I performed yeast two hybrid assay, which is an elegant method of investigating protein-protein interactions. Chriz cDNA was cloned into pGAD424 vector that contains a Gal4 Activation Domain (AD) . The full length Z4 cDNA was cloned into the pGBT9 vector, which contains the Gal4 DNA Binding Domain (DBD). Both constructs were proved by sequencing to be in frame with AD/BD domain respectively and cotransformed into SFY526 yeast cells. As a positive control the same cells were transformed with pCL1 vector, which contains the complete Gal4 cDNA. To test for self interaction cells were transformed with the plasmids BD-Chriz-FL and AD-Chriz-FL. For negative controls SFY526 cells were transformed with both the pGBT9 and

pGAD424 vectors only. To eliminate the possibility that BD-Z4 or BD-Chriz alone could result in a positive signal, SFY526 cells were cotransformed with BD-Z4 or BD-Chriz respectively with the pGAD424 vector. The colonies grown on selection plates were tested for their ability to express LacZ in a colony-lift filter assay (see Materials and Methods). In the presence of the X-gal substrate the yeast colonies become blue if they express proteins that interact with each other. Yeast cells containing BD-Chriz/AD or BD-Z4/AD, and the negative controls BD/AD, didn't show any staining (Figure 3.1A), whereas the positive controls did (data is not shown). Colonies containing AD-Chriz/BD-Z4 and AD-Chriz/BD-Chriz were positive in the colony-lift assay indicating that Chriz directly interacts with Z4, and interestingly Chriz also interacts with itself (Figure 3.1A). For quantification, the same assay was performed in solution, measured β-galactosidase activity was 1.94 (Units) for AD-Chriz/BD-Z4, 2.87(Units) for AD-Chriz/BD-Chriz (Table 3.1). These data indicate Chriz and Z4 interaction can be tested in this system, neither of them alone does show transcriptional activation.

Detected constructs	Interaction	β-Galacosidase activity (U)*
AD-Chriz/BD-Z4	+	1.94
AD-Chriz/BD-Chriz	+	2.87
AD/BD	-	0
AD/BD-Z4	-	/n.d.
AD/BD-Chriz	-	/n.d.

Table 3.1: Quantification of yeast two-hybrid interactions between Chriz and Z4 full length proteins. *Cells of the indicated genetic constitution were grown in liquid culture, lysed by freeze-thaw cycles using liquid nitrogen and -galactosidase activity was assayed using ONPG as a substrate.

3.1.2 Chriz fragment (from 279-768aa) is sufficient for interaction with Z4 in yeast

Next I wanted to know which part of Chriz is responsible for Z4 interaction. The Chriz protein has several conserved sections, most notably a conserved chromatin modifier domain, a chromodomain. The chromodomain has long

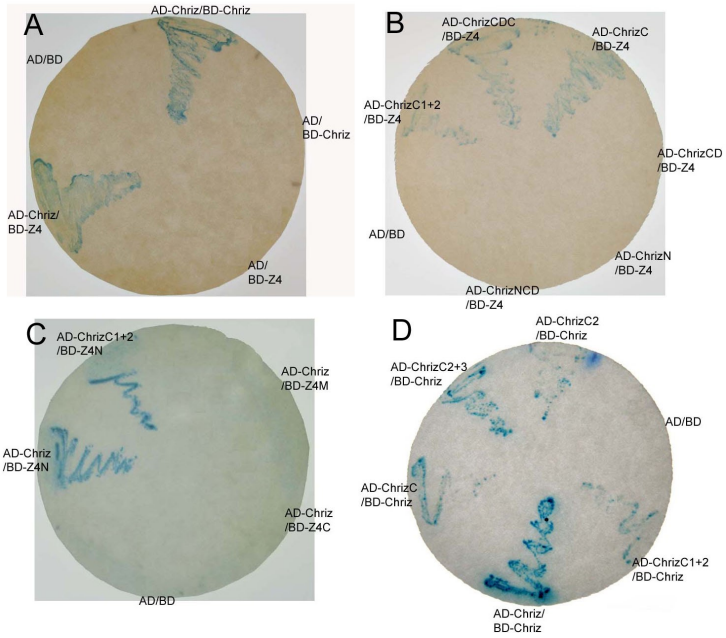

Figure 3.1: Yeast two-hybrid analysis of the Chriz and Z4 interactions and Chriz self interaction. Chriz and Z4 were cloned into the pGAD424 or pGBD9 vectors and cotransformed into the reporter yeast strain. Transformed yeast were transferred to filter paper soaked in X-gal. Blue color indicates interaction. Constructs used for cotransformation are indicated at the corresponding section.

been suspected to serve as a module mediating chromatin protein interactions in a variety of different contexts. For example, HP1 binds histone H3 at lysine 9 via its chromodomain (Bannister et al., 2001; Lachner et al., 2001). For sake of analysis the Chriz protein was divided into three parts, amino acids (aa) 1-212 upstream of the chromodomain, referred to as N-terminus, aa 206-286 the chromodomain (CD), and aa 273-926 downstream of the chromodomain referred to as C-terminus. As the chromodomain is quite short and by itself may not fold properly, the N-terminus plus CD (aa 1-286) and CD plus C-terminus constructs (aa 206-926) were used in this assay too. AD-ChrizN, AD-ChrizCD, AD-ChrizC, AD-ChrizNCD, AD-ChrizCDC constructs were cotransformed with BD-Z4 into yeast cells as before. The positive control was AD-Chriz/BD-Z4, the negative control was the vector combination AD/BD. Data showed that AD-ChrizC, AD-ChrizCDC interact with BD-Z4 (shown in Figure 3.1B), but the other fragments didnt (shown in Figure 3.1B). Therefore I conclude that the Chriz C terminus is the key part for the interaction. However, since the C terminus is still quite large (273 to 926aa), more than 600aa, so this part was further divided into ChrizC1 (aa 279-509), ChrizC2 (aa 500-768), ChrizC3 (aa 700-926), ChrizC1+2 (aa 279-768), and ChrizC2+3 (aa 500-926) respectively. Following transformation into yeast cells only with the C-terminal fragment, ChrizC1+2 did show interaction. According to quantification by β-galactosidase activity there was a significant interaction of this fragment with full length Z4 Table 3.2. In conclusion, though the chromodomain is a highly conserved, it is not required to bind Z4, whereas the C terminus is important for Z4 binding and the part (aa 279-768) is sufficient for Z4 interaction. The results of the Chriz-Z4 interaction are summarized in Figure 3.2.

3.1.3 Z4 N terminus interacts with Chriz

Z4 has seven zinc finger motifs within its central domain (aa 239-515). It strongly interacts with dsDNA (Eggert et al., 2004), but sequence specificity of binding has yet to be shown. The N terminal region (aa 1-237), upstream of this motif, can target the Z4 protein to interband regions on polytene

Detected constructs	Interaction	β-Galacosidase activity (U)*
AD-Chriz/BD-Z4	+	1.94
AD/BD	-	0
AD-ChrizN/BD-Z4	-	/
AD-ChrizCD/BD-Z4	-	/
AD-ChrizC/BD-Z4	+	0.40
AD-ChrizNCD/BD-Z4	-	/
AD-ChrizCDC/BD-Z4	+	0.18
AD-ChrizC1/BD-Z4	-	/
AD-ChrizC2/BD-Z4	-	/
AD-ChrizC3/BD-Z4	-	/
AD-ChrizC1+2/BD-Z4	+	0.15
AD-ChrizC2+3/BD-Z4	-	/

Table 3.2: Quantification of yeast two-hybrid interactions between Chriz fragments and Z4 full length protein. *Cells of the indicated genetic constitution grown in liquid culture, were lysed by freeze-thaw cycles using liquid nitrogen and β-galactosidase activity was assayed using ONPG as a substrate.

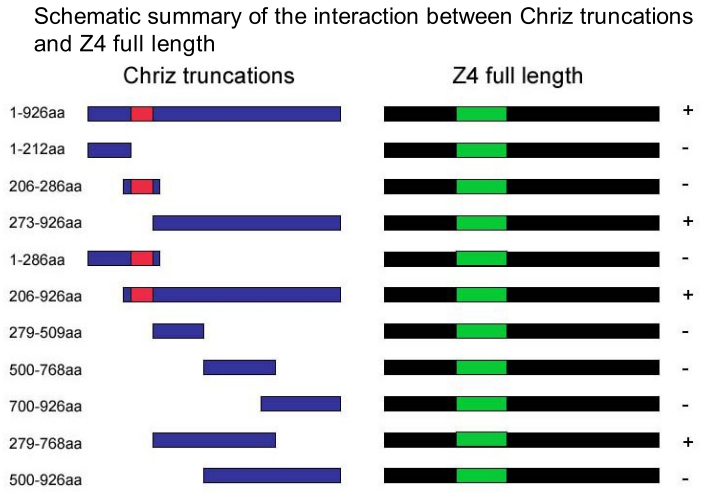

Figure 3.2: Summary of the interaction between Chriz and Z4. Blue bars represent Chriz protein, black bars represent Z4 protein, red represents the chromodmain, and green represents the zinc finger region. Chriz aa 279-768 is essential for interacting with Z4.

chromosomes (data not shown). As a next step I wanted to know which part of Z4 is responsible for binding to Chriz. Therefore, the following Z4 constructs were made, BD-Z4N (aa 1-237), BD-Z4M (aa 231-522), and BD-Z4C (aa 516-996) and cotransformed with AD-Chriz into yeast cells. In this assay the Z4 interaction domain for Chriz was mapped to the Z4 N terminal fragment (Figure 3.1 and Table 3.3). Then BD-Z4N and AD-ChrizC1+2 were tested for interaction. As expected these two parts bind to each other too. Measured β-galactosidase activity was 2.24 (Units) for AD-Chriz/BD-Z4N and 2.01(Units) for AD-ChrizC1+2/BD-Z4N. From these data, I conclude that the Z4 N terminus is sufficient for the interaction with Chriz. It also binds to the Chriz fragment aa 279-768 that was mapped as essential for interaction. Furthermore, the zinc finger motifs dont play a role in Z4-Chriz binding.

Detected constructs	Interaction	β-Galacosidase activity (U)*
AD-Chriz/BD-Z4	+	1.94
AD/BD	-	0
AD-Chriz/BD-Z4N	+	2.24
AD-Chriz/BD-Z4M	-	/n.d.
AD-Chriz/BD-Z4C	-	/n.d.
AD-ChrizC1+2/BD-Z4N	+	2.01

Table 3.3: Quantification of yeast two-hybrid interactions between Chriz full length and Z4 fragments. *Cells of the indicated genetic constitution were grown in liquid culture, lysed by freeze-thaw cycles using liquid nitrogen and β-galactosidase activity was assayed using ONPG as a substrate.

The results of determining the interaction domain of the Z4 protein are summarized in Figure 3.3.

3.1.4 Chriz fragment (500-768aa) can mediate Chriz selfinteraction

Interestingly, Chriz shows self-interaction, and this self-interaction judged by the measured β-galactosidase is stronger than Chriz-Z4 interaction. Therefore I wanted to map the fragments responsible for Chriz-Chriz interaction

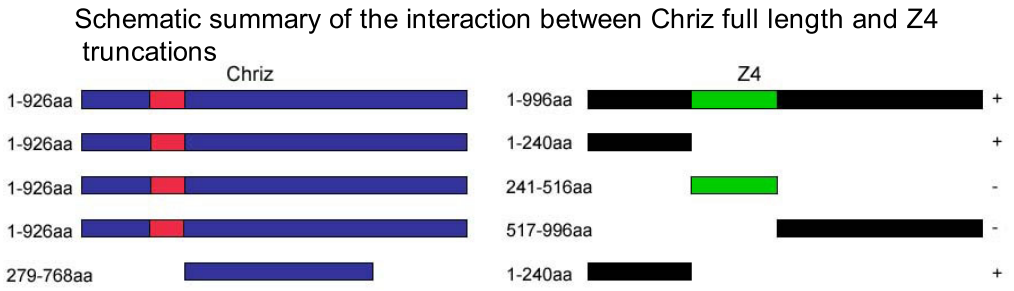

Figure 3.3: Blue bars represent Chriz protein, black bars represent Z4 protein, red represents the chromodmain, and green represents the zinc finger region. Z4 1-240aa is essential for Chriz interaction.

in more detail. The Chriz constructs described in 1.1 and 1.2 were co-transformed with BD-Chriz into yeast cells. Data showed that AD-ChrizC, AD-ChrizC2, AD-ChrizC1+2, AD-Chriz2+3 interact with BD-Chriz (Figure 3.1D). These results demonstrated that ChrizC2 (aa 500-768) is the domain for Chriz self-interaction. The interaction strength was measured in solution by β-Galactosidase activity and the data are shown in Table 3.4. The results of self interaction are also summarized in Figure 3.4.

Detected constructs	Interaction	β-Galacosidase activity (U)*
AD-Chriz/BD-Chriz	+	2.87
AD-ChrizN/BD-Chriz	-	/n.d.
AD-ChrizCD/BD-Chriz	-	/n.d.
AD-ChrizC/BD-Chriz	+	1.01
AD-ChrizC1/BD-Chriz	-	/n.d.
AD-ChrizC2/BD-Chriz	+	0.12
AD-ChrizC3/BD-Chriz	-	/n.d.
AD-ChrizC1+2/BD-Chriz	+	0.46
AD-ChrizC2+3/BD-Chriz	+	1.24

Table 3.4: Quantification of yeast two-hybrid assay for Chriz self-interaction. *Cells of the indicated genetic constitution were grown in liquid culture, lysed by freeze-thaw cycles using liquid nitrogen and βgalactosidase activity was assayed using ONPG as a substrate.

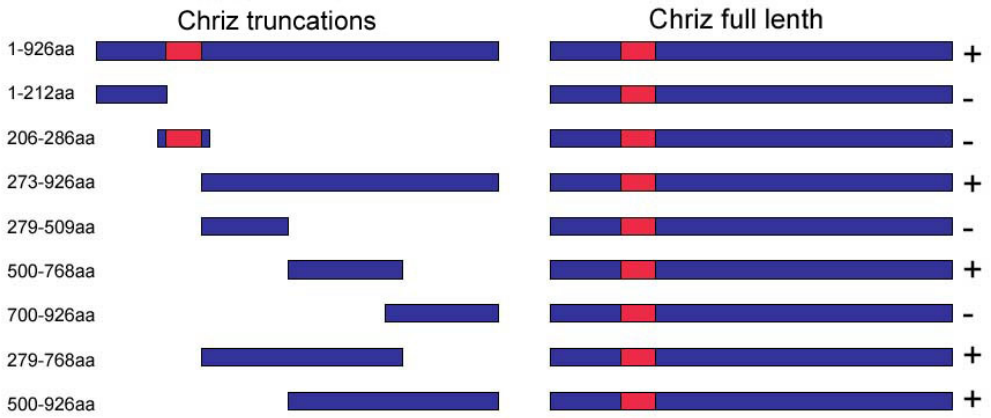

Figure 3.4: Self-interaction of Chriz fragments. Blue bars represents Chriz protein, red is the chromodmain.

3.1.5 Test of direct Chriz-Z4 interaction by pull down experiments

To confirm the interactions obtained in yeast and to demonstrate direct interaction between both proteins, a pull down assay was performed with GST fused to the Z4 N terminus and His-Myc tagged fusions of Chriz protein fragments. The fragments used, the Z4 N terminus (aa 1-237) cloned in pGEX-6p-1 and the Chriz fragments pMHchriz (aa 29-926), pMHchrizΔHindIII (aa 29-710), pMHchrizΔSphIHindIII (aa 29-291), pMHchrizΔSacIPstI (aa29-456), pMHchrizΔBamHI/PstI (aa29-346), and pMHchirzΔSmaI/pstI (aa 29-590) are illustrated in Figure 3.5. The Chriz pMH clones were obtained by Dr. A.Gortchakov (Gortchakov et al, 2005). Western blots of the protein extracts from transformed *E.coli* cells, were tested with anti-Myc antibody and showed the right molecular weight.(Figure. 3.6A). Purified GST-Z4 N-terminus and GST fusion were tested too (Figure 3.6B). Since all these proteins can be properly expressed in *E.coli*, these vectors can be used to perform the GST pull down assay. Z4-N-GST fusion proteins were purified and coupled to glutathione agarose beads as described in Materials and Methods. GST was used as a negative control. These beads were incubated with *E.coli* cell extracts derived from cells transformed with the Myc-His tagged

Chriz-constructs. After washing the beads the eluted proteins were analyzed on Western blots probed with antibodies to Myc. Figure 3.6A and Figure 3.6B showed the input of myc-Chriz-truncations, GST-Z4N and GST alone. Figure 3.6C shows that the Chriz (aa 29-926), Chriz (aa 29-710) and the Chriz (aa 29-590) fusion proteins were bound in this assay. These results demonstrate that the interaction between the Z4 N-terminus and Chriz is direct. Further, combining the results from the yeast and pull down assays I conclude, that the interaction is mediated by sequences in the C terminal domain of Chriz and the region of Chriz 456-590aa is necessary for their interaction.

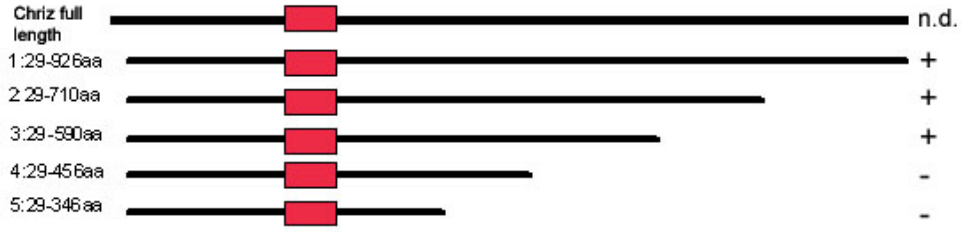

Figure 3.5: Schematic representation of Chriz derivatives tested for interaction with the Z4-N-terminus by the GST pull down assay. Red rectangles represent the chromodomain. Chriz aa 456-590 is necessary for interaction.

3.2 Genetic interactions between Chriz and Z4 alleles

To determine whether Chriz and Z4 genetically interact in vivo I explored interactions between mutant alleles of Chriz and Z4. Since Chriz and Z4 both are located on the third chromosome, I first combined three Z4 mutants over TM3, generating Z4 7.1/TM3, Z4 3.1/TM3 and Z4 1.3/TM3. Subsequently, I combined four Chriz mutants over TM3, generating ΔKG12/TM3, ΔKG6/TM3, ΔKG3/TM3 and KG26/TM3. Then Z4 7.1/TM3, Z4 3.1/TM3 or Z4 1.3/TM3 males were separately crossed with ΔKG12/TM3, ΔKG6/TM3, ΔKG3/TM3, ΔKG26/TM3 virgin females to generate Z4/Chriz transhe-

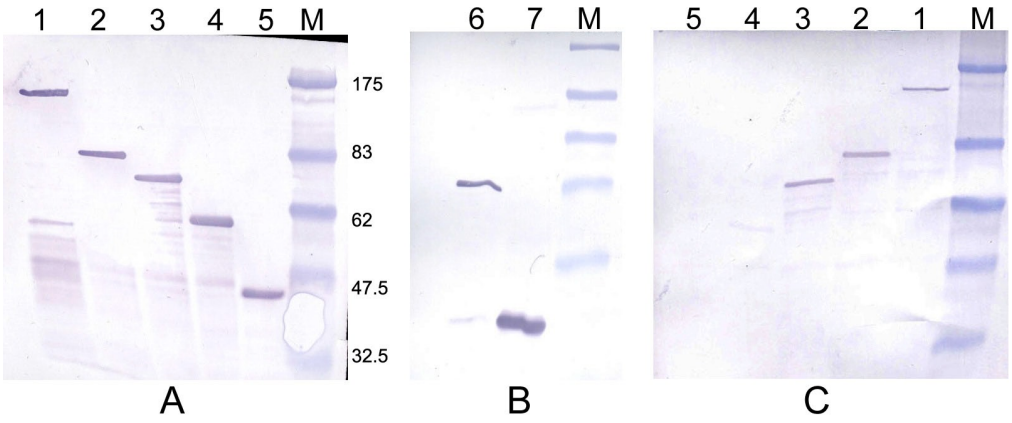

Figure 3.6: Myc-His-Chriz and Z4-N-GST fusion protein expression and pull down assay. A Immunoblot of input Myc-His tagged Chriz derivatives expressed in E.coli and tested by Western Blot. Lane 1 Chriz aa 29-926. Lane 2 Chriz aa 29-710, Lane 3 Chriz aa 29-590, Lane 4 Chriz aa 29-456. Lane 5 Chriz aa 29-346. B Immunoblot of the input Z4-N-GST-fusion protein and GST protein used for the pull down experiments detected with the anti-GST ab. Lane 6 Z4-N-GST, lane 7 GST alone. For pull down experiments, GST-Z4-N-GST was bound to glutathione-sepharose beads and incubated with the Chriz containing cell extracts to be tested. After washing, bound (C) proteins were analyzed by Western blot with Myc Ab. This defined Chriz aa 456-590 as necessary for mediating interaction. The relative migration of molecular size markers is indicated to the right of the immunoblots A; MWapp in kDa.

terozygous progeny. In these crossings the chromosome with TM3 balancer was identified by the Stubble bristles marker. Consequently, the experimental genotype could be distinguished by scoring non-TM3 balanced flies. The expected mendelian ratio of non-TM3 to TM3 adults would be 1:2 because TM3 homozygous animals are embryonic lethal. Table 3.5 shows that the non TM3 to TM3 ratio of each cross is about 1:2. This suggests that in a transheterozygous background a genetic interaction does not become apparent. The explanation for these data is that in the transheterozygous strains, one copy of each gene is still sufficient to perform the normal function. The genetic interaction can only be detected by recombination of both mutants alleles onto the same chromosome. However, since the genetic distance between both genes is rather short (estimated 0.1-0.3 cM) these experiments had to be postponed.

	$Z4^{7.1}$/TM3 (pupal lethal)[1]	$Z4^{3.1}$/TM3 (pupal lethal)[1]	$Z4^{1.3}$/TM3 (embryonic lethal)[1]
ΔKG12/TM3 (embryonic lethal)[1]	TM3:115 nonTM3:60	TM3:72 nonTM3:40	TM3:97 nonTM3:50
ΔKG6/TM3 (pupal lethal)[1]	TM3:93 nonTM3:51	TM3:66 nonTM3:33	TM3:109 nonTM3:64
ΔKG3/TM3 (embryonic lethal)[1]	TM3:70 nonTM3:41	TM3:80 nonTM3:40	TM3:62 nonTM3:32
ΔKG26/TM3 (larval lethal)[1]	TM3:100 nonTM3:61	TM3:97 nonTM3:49	TM3:128 nonTM3:68

Table 3.5: Genetic interaction crossing between Z4 mutants and Chriz mutants lines. [1] lethal phase of homozygous strains is indicated in brackets.

3.3 The activity of Chriz fragments are determined by complementation

3.3.1 overexpression of Chriz fragments

To determine the function of the different Chriz domains in vivo, I generated transgenic flies expressing various Chriz fragements under the control of

Gal4 promoter, that were tagged at the N terminus with a Myc-His epitope. Besides these Chriz derivatives, transgenic Chriz chromodomain point mutants were generated (Figure 3.7). Within the chromodomain there are three conserved β sheets and one α helical region. In order to identify the function of the highly conserved β sheets, I used overlap PCR to generate mutated chromodomains within Chriz (see Material and Methods). Two Chriz constructs were obtained. In the first construct, the first β sheet is mutated from PVEKI to AAVDD, in the second one, in all 3 β sheets the amino acids were changed from PVWKI YLVKW TWEVM to AAVDD DEETD AAFDD respectively. The DNA sequence of the constructs was confirmed by sequencing. These DNA fragments were cloned into pUAST-NLS-Myc-His vector and transformed into the germline by established methods. Transformants were selected and homozygous lines with single-copy inserts were established (shown in Table 3.6). The transgenes subcloned into the pUAST vector in Drosophila can be expressed by crossing these flies with fly strains transgenic for a particular GAL4 driver (Brand and Perrimon, 1993). In my study, I used mainly the GAL4-strains Gal4 231.1 and Gal4 SGS58 in which the expression of transgenes occurs preferentially in salivary glands during late embryo and larval stages. Transgenic Drosophila were investigated for the expression of Chriz derivatives after crossing with the GAL4-strain. Chriz expression in glands from 8 lines was analyzed on Western Blot, detected by Myc ab (Figure 3.7). Although at different strength, all of the Chriz derivatives were expressed at the expected molecular weight.

3.3.2 Complementation and dominant negative effects by the Chriz fragments

To assess the function of the mutant proteins in vivo, the transgenic lines were assayed for their ability to complement the embryonic lethal phenotype of ChrizΔKG3 under Gal4-T80 driver (Table 3.7). In contrast to the gland specific driver lines used before, Gal4-T80 is ubiquitously expressed. The crossing protocol is shown in Material and Methods.

The F3 generation has 4 potential genotypes for each transgenic con-

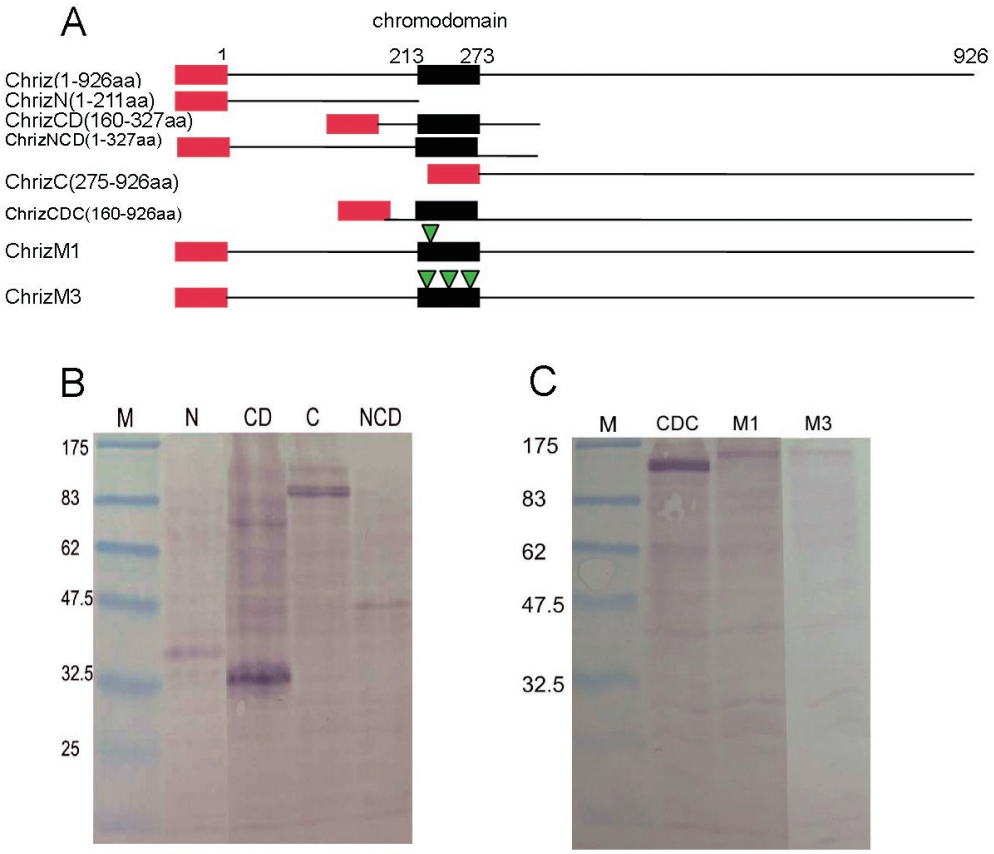

Figure 3.7: Expression of Chriz fragments and site directed mutations. Chriz constructs were expressed in vivo driven by either Gal4-231.1 or Gal4-SGS58. Protein extracts from salivary glands are detected by Western blot with Myc ab. A: schematic representation of Chriz domains for establishment of transgenic flies. Myc-His N-terminal tag (red bar) was used for constructs. Chriz N (aa1-211), Chriz CD (aa 160-327), Chriz NCD (aa1-327), Chriz C (aa 275-926) and Chriz CDC (aa 160-926). ChrizM1 (PVEKI mutated into AAVDD) and ChrizM3 (PVEKI mutated into AAVDD,YLVKW mutated into DEETD and TWEVM mutated into AAFDD) are also included. B: Expression of fragments N , CD, C, NCD, CDC and mutations M1 and M3 are indicated on top of the corresponding lanes. Protein marker is shown on the left side of each Western. The expected MWapp of each fragment: Chriz N is 31kD,Chriz CD is 27kD,Chriz NCD is 50kD, Chriz C is 80kD, Chriz CDC is 98kD, Chriz M1 and Chriz M2 is 110kD.

Transgenic fly line	Location	Homozygous viable[1]
pUASTmychis chrizN	N3/ 2^{nd}	Yes
pUASTmychis chrizCD	CD1/ 2^{nd},CD2/2^{nd},CD3/2^{nd}	Yes/all
pUASTmychis chrizC1	C1/ 3^{rd},C5/3^{rd},C6/2^{nd}	Yes/all
pUASTmychis chrizNCD	NCD1/3^{rd},NCD2/X, NCD4/3^{rd},NCD5/ 3^{rd}	n.d.,n.d.,Yes,n.d.
pUASTmychis chrizCDC	CDC2/3^{rd},CDC4/3^{rd}	No,Yes
pUASTmychis chrizmutant1	M1-1/3^{rd},M1-2/2^{nd},M1-3/2^{nd}, M1-4/3^{rd},M1-5/2^{nd},M1-7/3^{rd}	Yes,No,Yes,Yes,Yes,No

Table 3.6: List of all transgenic flies generated with above constructs. [1]Some of the transgenic animals are homozygous lethal. Since this varies for different insertions of the same construct this might be caused by the insertion into an essential gene disturbing its normal function.

struct:

$\frac{GalT80}{Cyo}i\frac{\Delta KG3}{TM6}$, $\frac{GalT80}{P}i\frac{\Delta KG3}{TM6}$, $\frac{P}{Cyo}i\frac{\Delta KG3}{TM6}$, $\frac{GalT80}{P}i\frac{\Delta KG3}{\Delta KG3}$

If the transgenic construct can rescue the Chriz embryonic lethality, then 25% of the flies that are homozygous mutants are non-Tubby. 75% are heterozygous for the Chriz mutantion as indicated by the presence of the balancer chromosome. If the transgenic fragment cannot rescue the embryonic lethality, only balancer chromosome bearing flies hatch. Since Chriz transgenes may also result in partial rescue, the Tubby marker on TM6 would allow the rescued larvae or pupae to be identified. At the same time, the $\frac{GalT80}{P}i\frac{\Delta KG3}{TM6}$ genotype may be scored to demonstrate if transgenic Chriz overexpression would induce lethality. Without such an effect the percentage of adult flies with $\frac{GalT80}{Cyo}i\frac{\Delta KG3}{TM6}$ and $\frac{P}{Cyo}i\frac{\Delta KG3}{TM6}$ should be equal. The number of flies of each genotype is listed in Table 3.7 and summarized in Figure 3.8. From these data, I conclude that the Chriz N terminus and CD (as expected) don't rescue the Chriz null mutant. However Chriz C can rescue up to the larval stage, pointing to a crucial function for this part of the protein. ChrizM1, M3 can rescue up to pupal stage. Chriz N and CD overexpression doesn't cause lethality. However both ubiquitous Chriz C, M1 and M3

overexpression strongly influence viability, although there are adult escapers. Phenotypically in either case some of the lavae rescued by Chriz C or Chriz M1 or M3 develop melanotic capsules.

	$\frac{GalT80}{Cyo}i\frac{\Delta KG3}{TM6}$	$\frac{GalT80}{P}i\frac{\Delta KG3}{TM6}$	$\frac{P}{Cyo}i\frac{\Delta KG3}{TM6}$	$\frac{GalT80}{P}i\frac{\Delta KG3}{\Delta KG3}$	Total num.
Chriz N	94	107	113	0	314
Chriz CD	137	151	162	0	450
Chriz C	175	5	187	33[1]	400
ChrizM1	213	16	241	108[2]	578
ChrizM3	271	0	251	133[2]	655

Table 3.7: Complementation and dominant negative effects of Chriz fragments; the number of animals and their genotypes are indicated. [1] rescued larvae only. [2] rescued pupae only.

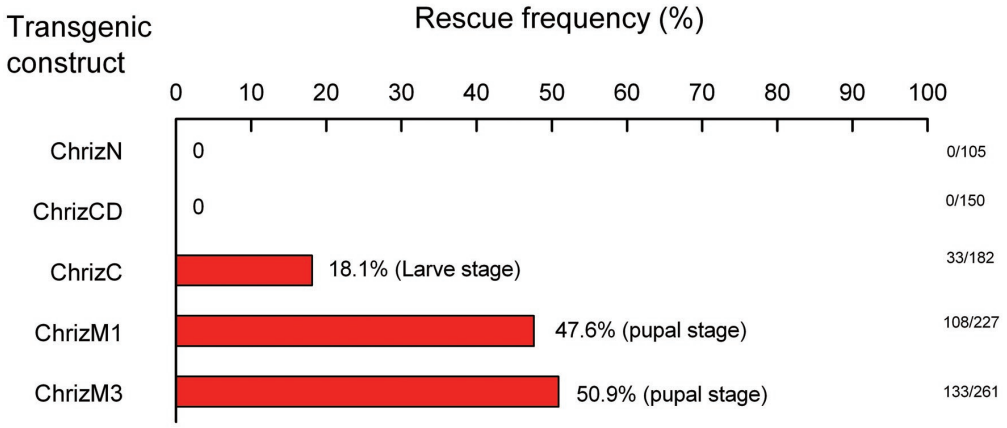

Figure 3.8: Complementation of ChrizΔKG3 lethelity by Chriz N, Chriz CD, Chriz C and Chriz M1and ChrizM3. The numbers of rescued flies were counted and the frequency is indicated for each genotype.

3.4 Identification of the domain for Chriz targeting to interband

Another question I could address using the established transgenic lines was, which part of the Chriz protein would be required for interband targeting.

Since the vector pUAST-NLS-Myc-His includes a nuclear targeting sequence, all expressed fragments will localize to nuclei. To identify interband targeting domains of Chriz, transgenic lines were crossed with gland specific driver line. Then larve were collected, polytene chromosomes of larval salivary glands expressing Chriz fragments were immunostained. Myc antibodies were used to detect the Chriz Myc-His fusion proteins. This analysis showed that the N terminus is sufficient to target the Chriz protein to interbands (Figure 3.9A). In contrast CD domain binds to bands (Figure 3.9B), a feature often found for non-specific binding proteins. The C terminus fragment has a slight preference for interbands (note: this fragment contains Chriz self interacting domain) (Figure 3.9C). The N terminus plus CD is also targeted into interband corroborating the importance of the N-term for interband targeting (Figure 3.9D). However, CD plus C terminus does not exclusively target into interbands (Figure 3.9E), suggesting that the self interaction induced targeting is not sufficient to overcome nonspecific binding induced by CD. Both forms of chromodomain mutated Chriz show clearly interband patterns (Figure 3.9F&G) demonstrating that the conserved β sheets of the CD are not required for interband specific binding. Therefore, I conclude that the Chriz N-terminus is solely responsible for interband targeting. Chriz interacts with Z4 by means of its C terminus in vitro and in vivo but it is likely that this interaction is not crucial for Chriz localization to interbands.

3.5 Effects of Chriz knockdown on chromatin protein binding

3.5.1 Chromosome phenotype of Chriz knocked down flies

Removal of Z4 by hypomorphic mutations results in a progressive loss of band/interband structure. Since a similar phenotype may be expected by the loss of its chromosomal binding partner Chriz, I assessed the consequences of loss of Chriz on polytene chromosome structure in interphase nuclei. Chro-

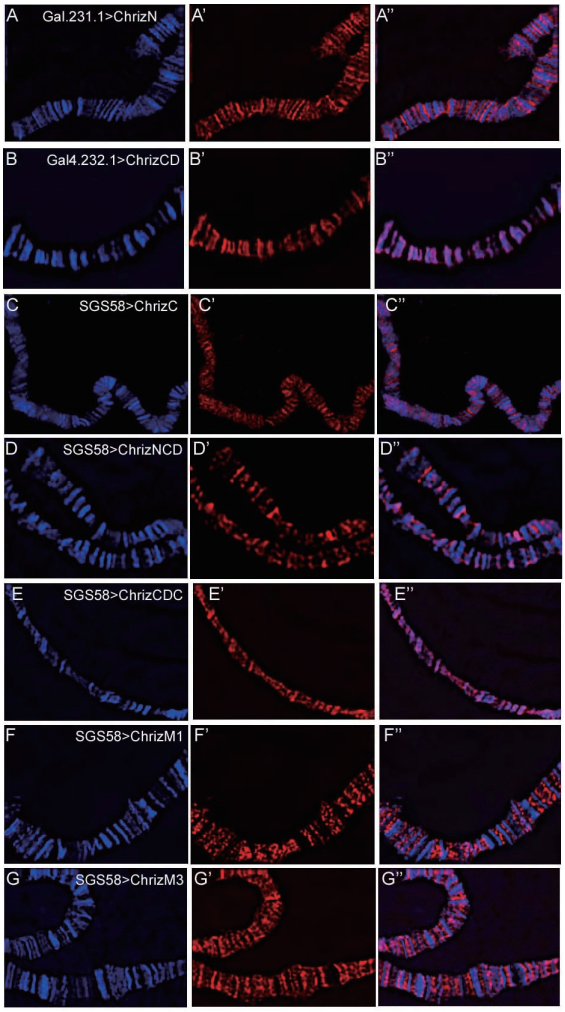

Figure 3.9: ChrizN terminus is required for targeting to interband. Polytene chromosomes of 3rd instar larvae expressing different Myc-His tagged Chriz fragments were labeled with DAPI (blue) and Myc ab (red) A: Polytene chromosomes from Chriz N (aa 1-211) larvae. B: polytene chromosomes from NCD (aa 160-327) animals. C: polytene chromosome from Chriz C (aa 275-926). D: polytene chromosome from Chriz NCD (aa 1-327). E: polytene chromosome from Chriz CDC (aa 160-926). F: polytene chromosome from ChrizM1. G: polytene chromosome from ChrizM3. Note: Chriz N is sufficient for targeting to interbands, and with CD together also shows interband binding. However, following deletion of the N terminus, neither ChrizCD, nor ChrizCDC binds to interbands. ChrizM1 and M3 interband binding suggests that the chromodomain is not required for interbands specific binding.

mosomal squashes prepared from either wild-type or Chriz RNAi mutant larvae were performed. Chriz RNAi was induced by Gal4231.1 driver line. Chromosomes were labeled with Hoechst to visualize the DNA and with Chriz antibody. Overall, the gland size of RNAi animals was only 1/3 of that of wild type glands. In contrast to the regular Hoechst stained bands seen in wild-type polytene chromosomes, this pattern becomes severely perturbed in Chriz RNAi larvae (Figure 3.10). The chromosome became lighter and lost band interband distinction in general although locally there is still some banding pattern. Thus, these results suggest that like Z4, the Chriz is involved in establishing or maintaining the normal interphase chromosome structure.

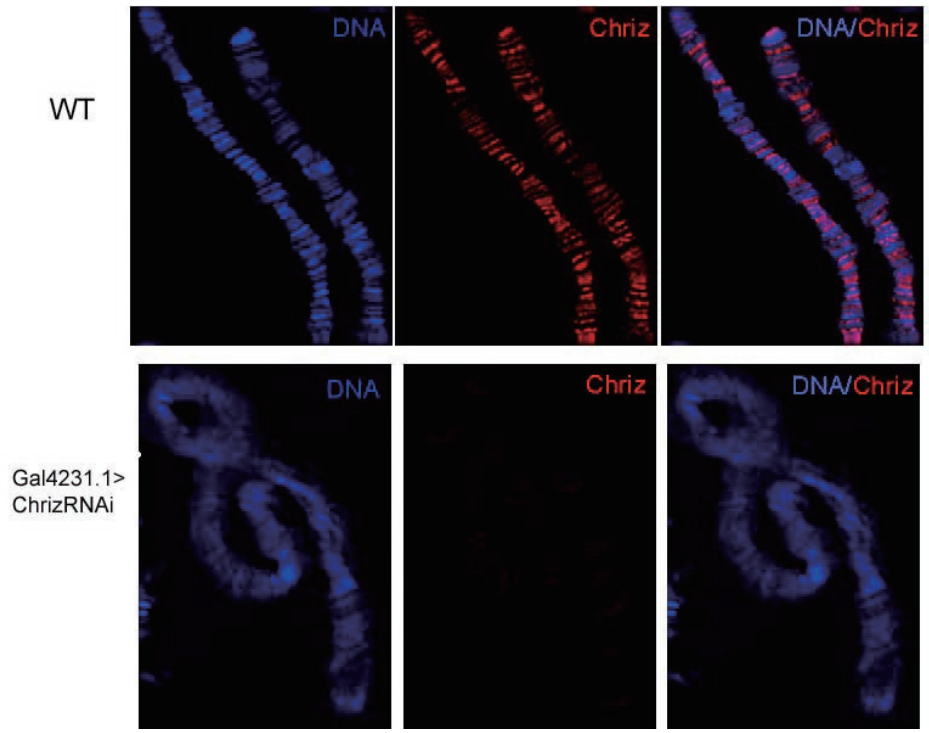

Figure 3.10: Chromosome phenotype of ChrizRNAi. Polytene chromosomes of 3rd instar larvae of WT and ChrizRNAi lines were double labeled with DAPI (blue) and Chriz ab(red) A: polytene chromosome from WT B: Polytene chromosome from Chriz RNAi lines induced with Gal4 231.1 line.

3.5.2 Z4 protein binding following ChrizRNAi knockdown

Z4 binding

The immediate question resulting from the previous section is, how to mechanistically explain the loss of chromosome structure induced by Chriz RNAi. Conceivably, this could be induced by loss of binding or other chromosomal proteins or by a loss of specific chromatin modifications. Since the coimmunoprecipitation and yeast two hybrid assay showed Chriz and Z4 proteins directly bind to each other, it was suggestive to test whether the chromosomal binding of Z4 was dependent on Chriz. To this end, I used Chriz RNAi-induced knockdown driven by the Gal4 231.1. As shown in Figure 3.11, Chriz staining in such animals is strongly reduced compared to WT. The Z4 staining in interbands is reduced obviously too (Figure 3.11). However, I want to emphasize that there is still some Chriz protein present in interbands. So I conclude that the chromosomal binding of Z4 is dependent on Chriz.

Vice versa, Z4 RNAi lines were crossed with the same driver line Gal4 231.1 to induce gland specific knock down. Polytene chromosomes from such animals showed a strongly reduced staining with Z4 antibodies compared to the WT staining (Figure 3.12). But on staining with Chriz antisera, the staining intensity is similar to that in wild-type (note that the images for Z4 in Figure 3.11 and for Chriz in Figure 3.12 respectively were taken and reproduced under exactly the same conditions). This suggests that chromosomal binding of Chriz does not depend on Z4.

Chriz RNAi and Z4 RNAi induction in wing discs

To confirm the above observation in diploid cells, I used the wing disc specific driver line engrailed-Gal4 in combination with Chriz RNAi and Z4 RNAi flies to knock down these proteins in wing disc cells. Wing discs were prepared from the third instar larvae of such animals and stained with Z4 ab and Chriz ab. In the posterior region of the disc, where RNAi induction takes place

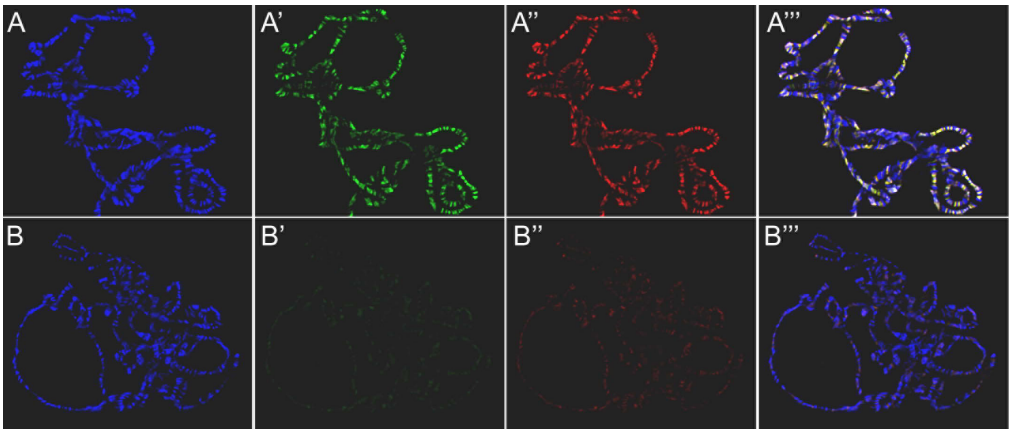

Figure 3.11: Z4 interband targeting: polytene chromosomes from salivary glands of Chriz RNAi-induced animals were stained with DAPI (blue), Z4 antibody (red) and Chriz antibody (green). A is chromosomes from WT. B is chromosomes from Chriz RNAi induced animals. Note, when Chriz protein is reduced, then the Z4 protein level also declines.

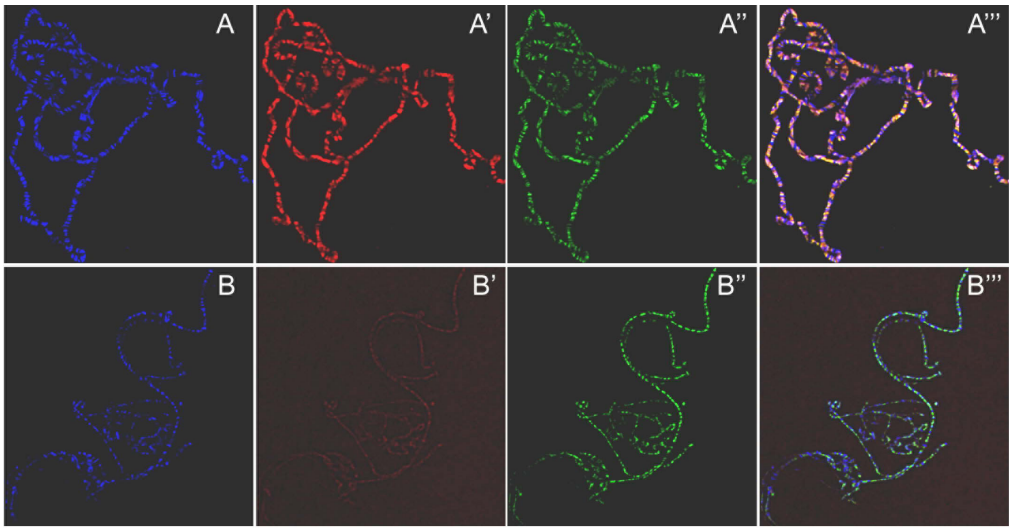

Figure 3.12: Chriz interband targeting: polytene chromosomes from salivary glands of Z4 RNAi-induced animals were stained with DAPI (blue), Z4 antibody (red) and Chriz antibody (green). A is chromosomes from WT. B is chromosomes from Z4 RNAi induced animals. Note, when Z4 protein is reduced, Chriz protein level remains still quite normal.

due to the engrailed driver line, Chriz protein was strongly reduced (Figure 3.13B), Z4 protein level was also induced (Figure 3.13C), however not as strongly as Chriz, since residual Z4 staining (green) is a parent in the overlay picture in Fig.12. Vice versa, when Z4 protein was reduced by RNAi, Chriz protein level was normal. Also the reduction of Z4 in ChrizRNAi animals has still to be interpreted. I conclude that Z4 protein binding or stability is dependent on Chriz, but Chriz binding or stability does not depend on Z4 (Figure 3.14).

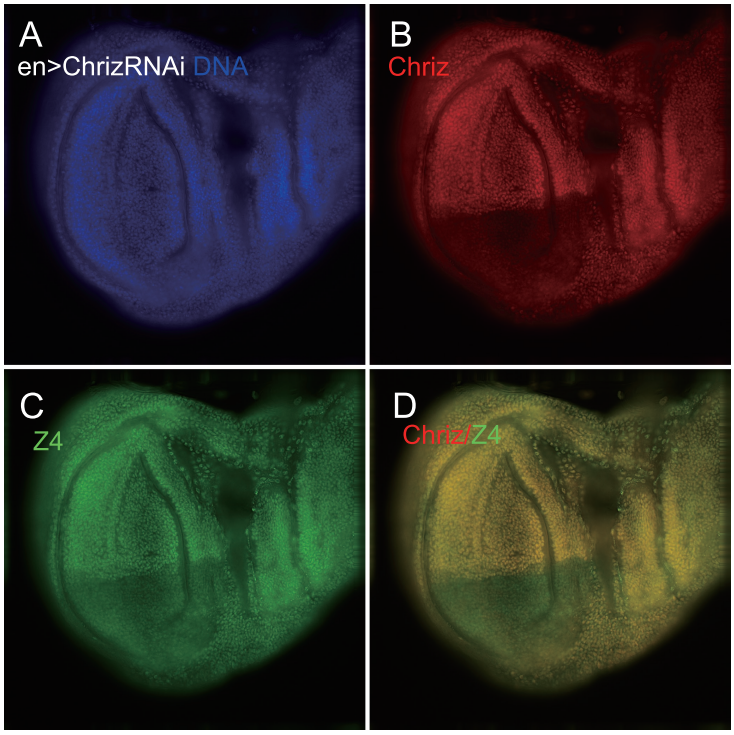

Figure 3.13: Knockdown of Chriz does affect Z4 distribution. Chriz RNAi line was crossed with the engrailed Gal4 driver line, which results in Chriz reduction in the posterior region of imaginal discs. Immnunofluorescence staining of third instar larval wing discs with DAPI and Chriz and Z4 antibodies. A: DAPI staining. B: Chriz antibody staining in the posterior region. Chriz is strongly reduced. C: Z4 pattern in the posterior region. Z4 is also reduced. D: Z4 and Chriz double staining.

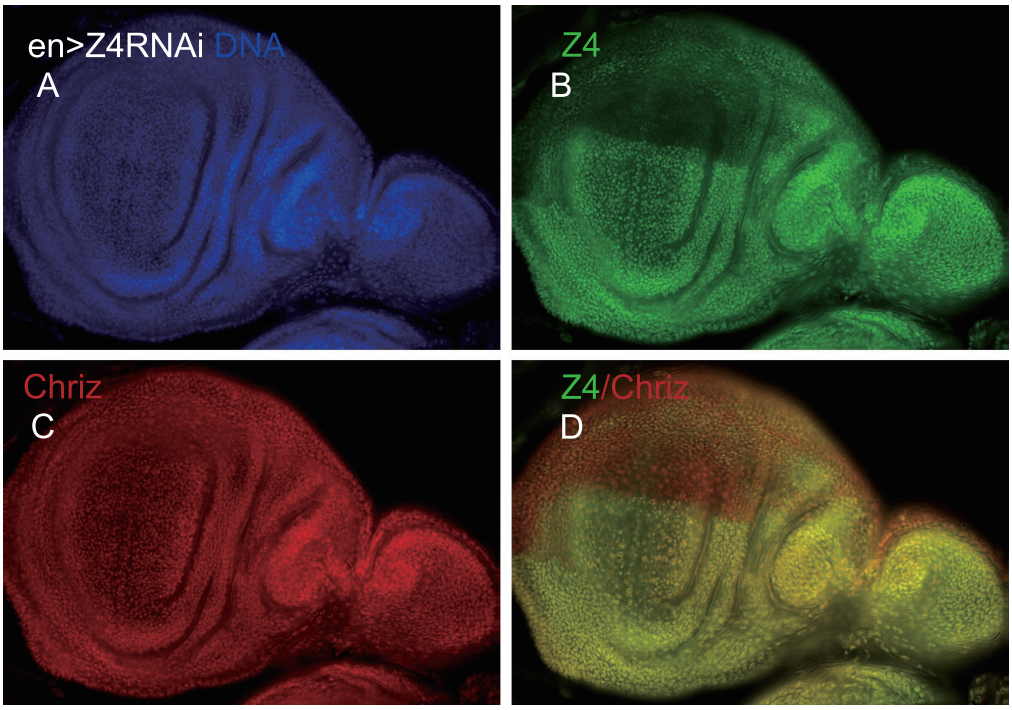

Figure 3.14: Knockdown of Z4 protein does not affect Chriz binding. The Z4RNAi strain was crossed with a strain containing an engrailed Gal4 driver, which resulted in Z4 reduction in the posterior region. Immnunofluorescence staining of third instar larval wing discs by DAPI and Chriz- and Z4-antibodies. A: DAPI staining. B: Z4 ab staining; at the posterior region Z4 protein is knocked down. C: Chriz ab staining. Note that in the posterior region Chriz is present in normal amount. D: Z4 and Chriz ab double staining.

3.5.3 Jil-1 and Histone 3 phosphorylated at S10 (H3-pS10) levels are decreased in ChrizRNAi lines

Jil-1 is a tandem kinase which specifically phosphorylates histone 3 serine10 at interphase (Wang et al., 2001). Jil-1, and likewise H3S10 phosphorylation are enriched in interbands of polytene chromosomes.There is evidence that this type of histone modification maybe connected to the decondensed chromosomal state. We and others already showed by immunoprecipitation, that Jil-1 coprecipitates with Chriz ((Rath et al., 2006); H. Saumweber, unpublished) and Z4 (H.Saumweber, unpublished); Jil-1 also colocalizes to a large extent with Chriz and Z4 proteins on polytene chromosomes, indicating a close interaction between these 3 proteins. To further investigate this interaction, I determined Jil-1 chromosomal binding and H3S10 phosphorylation in a ChrizRNAi background to see if Chriz will affect Jil-1 interband localization and H3S10 phosphorylation. Therefore I first performed immunostaining of polytene chromosomes of Chriz RNAi inducing with Gal4 231.1 animals with Chriz-, Jil-1-, H3pS10- antibodies. As illustrated in Figure 3.15, Jil-1 abundance on chromosomes is reduced compared to WT under those conditions. In the same genetic background phosphorylated H3S10 is dramatically reduced (Figure 3.16).

In addition, the same experiment was done with Z4 RNAi line. But no obvious change is detected (data is not shown). To confirm and extend these observations, the protein levels of Jil-1 and H3pSer10 were determined by immunoblot analysis of salivary gland from wild-type, Chriz RNAi and Z4 RNAi mutant animals. Western blot were probed with anti- H3pS10, anti-Chriz, anti-Jil-1, and anti-histone H3 antibodies. Anti-lamin antibodies were used as a loading control. As illustrated in Figure 3.17, Chriz RNAi animals show lower levels of H3pS10 when compared to wild-type larvae. In the loading controls, the levels of histone H3 and lamin were roughly equivalent to wild-type.

Chriz RNAi lines show a strongly reduction of the Jil-1 protein. Interestingly, ChrizRNAi shows a reduction of Jil-1 protein whereby the remaining protein is shifted in its apparent molecular weight by 20-30kD. Z4 RNAi in-

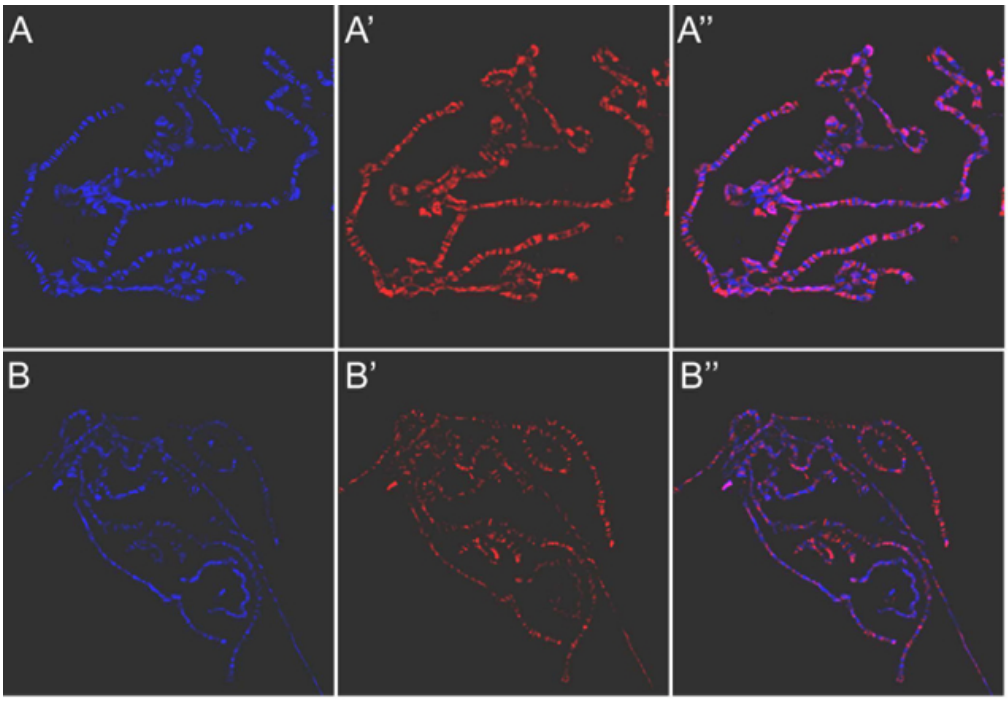

Figure 3.15: On Chriz RNAi induction Jil-1 levels decline. Polytene chromosomes of 3rd instar larvae of WT and Chriz RNAi lines were double stained with DAPI (blue) and Jil-1 ab(red). Compared to WT (A), Jil-1 ab staining is mildly reduced in ChrizRNAi animals (B). ChrizRNAi was induced with Gal4-231.1 at room temperature.

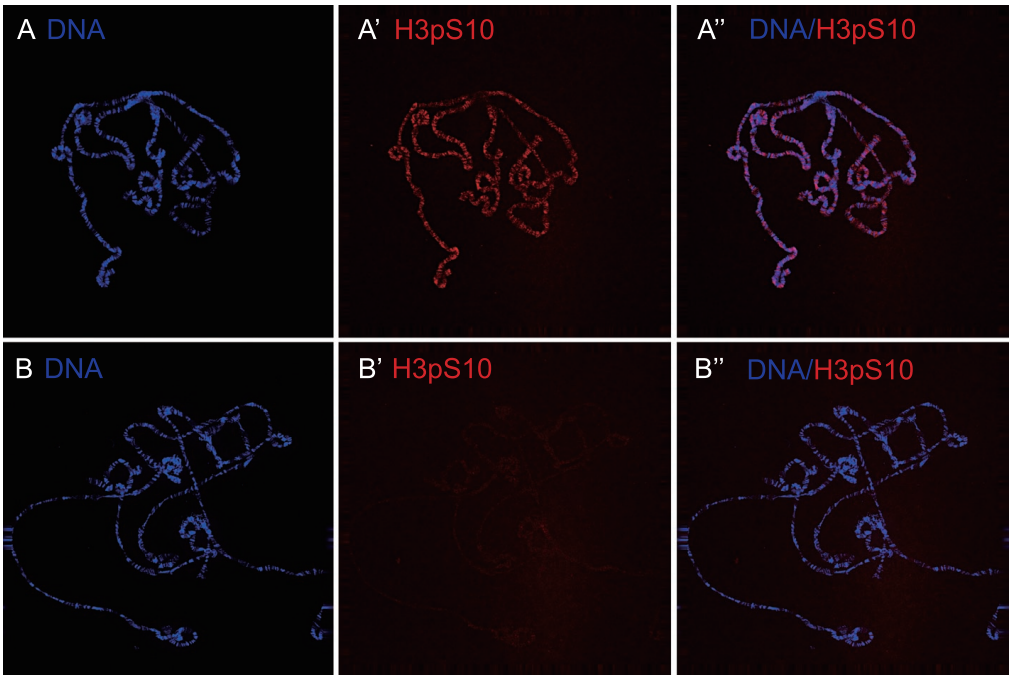

Figure 3.16: H3pS10 is reduced by Chriz protein knock down. Polytene chromosomes of 3rd instar larvae of WT and ChrizRNAi lines were double labeled with DAPI (blue) and H3pS10 ab(red) A: polytene chromosomes from WT B: Polytene chromosomes from Chriz RNAi lines induced with Gal4 231.1 at 28°C. Compared to Wild type H3pS10 level was dramatically reduced in ChrizRNAi line.

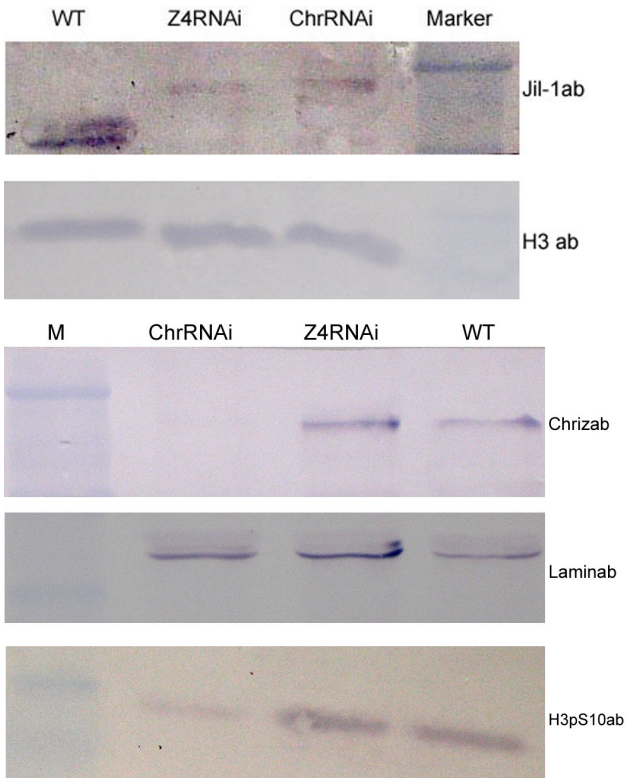

Figure 3.17: ChrizRNAi results in decreased phosphorylation of the Histone H3Ser10. Immunoblots were performed on extracts from WT, Chriz RNAi and Z4 RNAi glands and compared to wild-type. The immunoblots were incubated with Chriz ab, with phospho-histone H3 S10 ab (H3pS10) and Jil-1 ab as indicated. Lamin ab and histone H3 ab were used as loading controls. Note the shift of remaining Jil 1 in MWapp following Chriz- and Z4 RNAi.

duction results in a similar effect, also shifting the molecular weight of Jil-1. In summary, on Chriz protein knockdown, both Jil-1 and H3pS10 levels are significantly reduced. Therefore, I conclude that H3S10 phosphorylation is dependent on proper Chriz expression.

Chapter 4

Discussion

4.1 Chriz interactors

4.1.1 The Chriz protein is a central element of a chromatin complex located in interbands

Previous experiments by coimmuniprecipation and colocalization suggested that the interband proteins Chriz and Z4 are present in a common chromatin complex. In this work I demonstrate that this is the case and that both proteins directly interact. As shown by yeast two hybrid and pull down assays using fragments of the Chriz and Z4 proteins respectively the putative interaction domains were narrowed down and it is shown that Chriz (aa 279-768) and Z4 N (aa 1-240) fragments are sufficient for mutual interaction. Both the Chriz chromodomain and the Z4 zinc finger domain, although very conserved motifs, do not mediate Chriz and Z4 interaction. Of course, the Chriz fragment (aa 279-768) is still quite large. However, since GST pull down demonstrated that Chriz (aa 29-590) could still interact with Z4 N, but Chriz (aa 29-456) could not suggests that Chriz (aa 456-590) is necessary to mediate the interaction between these two proteins. Whether this fragment is also sufficient is presently under investigation.

In addition to Z4 binding, the C-terminal region provides binding sites for other chromatin proteins as well. Chriz (aa 500-768) for instance mediates

self interaction. The significance of Chriz self interaction in vivo is unclear at present. Conceivably, it may support recruitment or stabilize Chriz binding on chromosomes. We and others have shown that Chriz also interacts with Jil-1, a trandem kinase (Rath et al., 2006); H. Saumweber, unpublished). Jil-1 kinase activity is equired for H3S10 phosphorylation during interphase. Interestingly, Jil-1 also interacts with Z4 (H. Saumweber, unpublished). All three proteins colocolize in interbands of polytene chromosome. However, in contrast to Chriz and Z4 that are perfectly colocolized (maybe in a stoichiometric complex), colocalization with Jil-1 is not 100%. Jil-1 may be more dynamically associated or it may be involved in other complexes function, besides in the Chriz/Z4 complex, for example, in dosage compensation (Jin et al., 1999). It was shown that the Jil-1 C terminal acidic region (aa 887-1033) interacts with the Chriz C terminus (Rath et al., 2006). However, this was not further mapped. Since the Chriz C terminal region (as defined operationally) is quite large it may mediate different protein interactions simultaneously. It is clear that in the complex Chriz directly interact with Z4 and Jil-1 but we still do not know if Z4 directly interacts with Jil-1. In addition to these studies, Chriz was shown to interact with the spindle matrix protein Skeletor (Walker et al., 2000; (Rath et al., 2004); Chriz was called Chromator in their papers).The interaction with Skeletor is direct since it was detected in a yeast two hybrid screen and subsequently confirmed by pull down assays (Rath et al., 2004). Immunocytochemical labelling of Drosophila embryos, S2 cells and polytene chromosomes demonstrated that the two proteins show extensive colocalization throughout the cell cycle although their distribution is not identical (Rath et al., 2004). During interphase, on polytene chromosomes, Chriz is localized to interband chromatin regions in a pattern that overlaps that of Skeletor. During mitosis both Chriz and Skeletor detach from the chromosomes and align together in a spindle-like structure with Chriz additionally localized to centrosomes that are devoid of Skeletor (Gortchakov et al., 2005). The extensive colocalization of the two proteins is compatible with a direct physical interaction between Skeletor and Chriz.

4.2 Chriz is responsible for targeting the complex to interbands

Chriz like Z4 antiserum stains a speckled pattern of many sites in Drosophila Kc-cell nuclei, that are neither correlated with condensed chromatin nor with sites of hnRNP binding proteins and on polytene chromosomes Chriz is specifically targeted to interbands (Gortchakov et al., 2005). Interbands containing losely condensed chromatin are formed in a reproducible pattern. The maintenance of this pattern is dependent on the presence of Z4 (Eggert et al., 2004). Since Z4 is a close Chriz interactor I became interested in the chromosomal targeting mechanism of both proteins to learn more about the binding of the complex. To address this question, different fragments of Chriz were ectopically expressed as myc tagged Chriz fusion proteins that can be selectively detected by the Myc antibody in the presence of the endogeneous protein. I found that Chriz N alone can bind to interbands like the full length protein. In contrast, the Chriz chromodomain is not involved in interband targeting since nonspecific binding to bands was observed only. This is supported by the two Chriz constructs mutated within their Chromodomain that still specifically bind to interbands. When ChrizN was fused with ChrizCD the resulting ChrizNCD also shows interband binding consistent with a targeting function of the N-terminus. Chriz C alone shows both band and some interband binding that is explained by the self-interaction site that targets this fragment to the chromosomal sites of the endogenous Chriz superimposed by nonspecific binding to bands. When ChrizCD was fused with ChrizC the resulting ChrizCDC covers the whole chromosome, both bands and interbands. Apparently the nonspecific binding of this fragment is enhanced by the presence of the chromodomain. From these data, I conclude that Chriz N (aa 1-211) is crucial for Chriz targeting into interbands.

It appears, that Chriz is central for targeting other proteins to interbands. Recently we found that the Z4 N terminus, rather than Z4 zinc finger motif is responsible for Z4 interband targeting (H. Eggert, unpublished). Since the same Z4 fragment mediates Chriz binding, the interaction with Chriz could

be the mechanism for Z4 targeting to interbands. In fact, when Chriz protein is reduced by RNAi, then the Z4 level also is reduced. Vice versa, when Z4 protein is reduced, Chriz protein level is still quite normal. If Z4 is targeted to interbands by Chriz interaction only, I predict that overexpression of the Z4-N terminal fragment should suppress endogenous Z4 binding. Interestingly, this is exactly what was observed recently in our group (S. Moebus, personal communication). Jil-1 interband targeting was not yet investigated. However, recent experiments expressing a tagged version of Jil-1 suggest, that its chromosomal binding is mediated by protein-protein interaction. Since it coprecipitates with Chriz and Z4 and its C terminus (aa 887-1033) can interact with Chriz (Rath et al., 2006), I assumed that Jil-1 is targeted to interbands largely by Chriz interaction. This is consistent qualitatively with the observed reduction of Jil-1 binding to polytene chromosomes observed following Chriz RNAi knockdown. However, there is still significant chromosomally bound Jil-1 under these circumstances. Either, following RNAi there is still sufficient Chriz left for binding or Jil-1 is tethered to chromosomes by an additional as yet unidentified mechanism (see discussion below).

There are still many open questions: first, how Chriz target into interbands? A DNA sequence specific binding is less likely since I could not detect any DNA binding motif within the Chriz N-terminal fragment. Another possibility is an interaction with a chromosomal protein that still has to be identified. From the many interband binding proteins in Drosophila, dMBD-R2 was found to be exactly colocalized with the Chriz/Z4 complex (H. Saumweber, unpublished). However, a direct interaction of these proteins remains to be shown. Currently, experiments in our group are in progress to isolate the Chriz/Z4 complex by affinity methods using Tap-tagged versions of Chriz and Z4 proteins. Summarizing these data, I propose that Chriz is first bound to interbands by an as yet unknown mechanism, then other proteins like Z4 and Jil-1 are targeted to interbands by interaction with the Chriz C terminal domain. Z4 interband targeting depends on Chriz protein.

4.3 Chriz and the function of the complex

4.3.1 The Chriz chromodomain

Chriz, like Z4, is an essential protein. Lack of the protein is late embryonic lethal. Though this has to be tested, the embryos probably survive only due to the maternally provided Chriz protein. An important feature of the Chriz protein is the presence of a chromodomain. The chromodomain is essential for Chriz function since site directed changes within this domain failed to rescue or only partially rescued genomic Chriz mutations. The function of most chromodomain proteins identified so far has been related to the establishment or maintenance of a variety of chromatin conformations Cavalli and Paro (1998); Brehm et al., 2004). For instance the chromodomains of Heterochromatin protein 1 (HP1) and Polycomb (PC) mediate the specific recognition of methylated lysine residues K9 and K27 of histone H3, respectively (Nielsen et al., 2002); Fischle et al., 2003). Furthermore, this motif can mediate protein-RNA interaction, like in case of MOF (Akhtar et al., 2000), or protein CDNA interactions, like observed for Mi-2 (Bouazounek et al. 2002).Comparison of the Chriz chromodomain sequence with those of HP1 and PC reveals significant conservation in residues that define the spatial organization characteristic of the chromodomain module . Nevertheless, two out of the three aromatic residues in chromodomain of HP1/PC that were identified to be crucial for the recognition of histone H3 di- and trimethyllysine are substituted in the chromodomain of Chriz for charged amino acids. It was suggested that these substitutions would rule out Chriz binding to H3K9 di- and trimethylysine. However, assays for the direct targets of the Chriz chromodomain still have to be performed. Whatever its function, the chromodomain of Chriz does not mediate Chriz/Z4 interaction, nor does it mediate Chriz interband targeting into interbands.

4.3.2 Chriz is required for maintainance of the chromosome structure

As previously shown by our group, downregulation of the Chriz interactor Z4 by hypomorphic mutations greatly affects chromosome structure, resulting in a loss of band interband discrimination. A similar effect is expected as a consequence of Chriz downregulation and it would be of considerable interest to understand the cause of such an effect. In order to address this question, I used inducible Chriz RNAi lines generated by using pUAST as vector to knock down endogenous Chriz protein in different tissues during development.

The reduction of Chriz in salivary glands affects the polytene chromosome structure similar to Z4 hypomorphs. In ChrizRNAi mutant polytene chromosomes the chromatids were loosely paired and chromosomes became fragile during squash preparation although in certain regions band/interband sections still can be detected, probably caused by the incomplete elimination of Chriz by RNAi. In some cases I also observed the formation of small glands containing small, condensed and coiled chromosomes. These findings suggest that Chriz function is required for maintaining the pattern of bands and interbands in polytene chromosomes. This observation is consistent with results of Rath et al.(2006), that generated hypomorphic Chriz mutants by EMS-induced point mutations. The combination of specific alleles created transheterozygotes that survived to third-instar larval stages and allowed the analysis of their effect on polytene chromosome structure. Rath et al (2006) reported that the mutant chromosomes were disrupted and the chromosome arms were coiled and condensed. Another feature of the phenotype was the folding of the chromosomes with numerous ectopic contacts connecting non-homologous regions. However, despite these disruptions, distinct band and interband regions were still clearly discernable in the mutant chromosomes (Rath et al., 2006). The reasons for the slight differences in phenotype might be differences in the extent of Chriz protein elimination by both approaches. In my case the Chriz RNAi line was generated by cloning Chriz N terminal (1-456aa) in sense and antisense orientation into pUAST, so that Chriz will

be interfered from transcription start site. In the transheteozygous hypomorphic Chriz mutations used by Rath et al (Rath et al., 2006), one allele would translate the first 71aa, the other allele the first 612aa. The second allele still could be partially functional since the mutation would produce a truncated Chriz protein that still has the Chriz N terminal, the chromodomain and part of the C terminus possibly containing the Z4- and self interacting domains. Therefore, the protein level and the protein fragments that are present in RNAi or transeterozygous mutant flies may be quite different, which may contribute to the phenotypic difference. Z4 hypomorphic mutations have a quite similar phenotype as the Chriz RNAi line. This suggests that Z4 is essentially required for the normal function of the complex. Be it for reasons of less Z4 binding (in Chriz RNAi animals) or for diminished Z4 protein abundance (in Z4 hypomorphs), the complex would be defect.

Since we and others observed an interaction of the kinase Jil-1 with the Chriz/Z4 complex as well, it may be relevant to compare here also the chromosomal phenotype of Jil-1 mutations (Deng et al., 2005). For Jil-1 hypomorphic alleles, the phenotype of autosomal polytene chromosomes is a misalignment of interband chromatin fibrils and increased ectopic contacts between nonhomologous regions. These observations were obtained by TEM microscopy at a resolution of about 20 nm. Since the resolution of fluorescence microscopy is about 200 nm. I can not directly compare my results, but I would not exclude the possibility that Chriz RNAi phenotype is caused by misalignment of interband chromatin fibrils. In contrast, coiling of the male X polytene chromosome was not observed in Jil-1 mutants. Instead, the shortening of the male X chromosome appeared to be caused by increased dispersal of the chromatin into a diffuse network without any discernable banded regions (Deng H et al., 2005). Such a phenotypic difference between sex chromosomes and autosomes was not observed for Chriz RNAi animals. Since Jil-1, but not Chriz, is enriched on the X chromosome it may have some extra function there.

4.3.3 Chriz is required for maintenance of Jil-1 activity in interbands

In contrast to my expectation, there was still significant Jil-1 binding on polytene chromosomes of Chriz RNAi animals. However, by Western blot, there is either no Jil-1 detected in Chriz RNAi animals or a Jil-1 band that is shifted by 20-30kD to a larger molecular weight compared to wildtype. This experiment was repeated with similar results several times. This suggests that after Chriz knock down Jil-1 experiences some posttranslational modification and is destabilized. Therefore, normally binding of Jil-1 in the Chriz/Z4 complex may mask Jil-1 for degradation and/or stabilize the Jil-1 activity. It is possible that the modified and inactivated form of Jil-1 is still bound to polytene chromosomes for some time explaining the observed moderate reduction in immunofluorescence staining.

Although this has to be investigated, the protein modification causing the shift in molecular weight could be polyubiquitination. Ubiquitin is a peptide that occurs in all eukaryotic cells. It is attached to lysine residues of proteins as single or multiple copies with the major role in modifying the function of proteins or to mark these proteins for proteolysis. Several ubiquitin molecules have to be attached to a lysine residue on the protein to be degraded, in a process called polyubiquitination.The protein then is routed to a proteasome, a barrel-shaped multisubunit protein complex where the proteolysis occurs.

Ubiquitin consists of 76 amino acids and has a molecular mass of about 8.5 kDa. It is highly conserved among eukaryotic species. Since human and yeast ubiquitin share 96% sequence identity. Addition of ubiquitin to target molecules is a omplex process involving ubiquitin activating enzymes, ubiquitin conjugating enzymes and ubiquitin ligases (reviewed by (Pickart, 2001)). Often, ubiquitin molecules are further added on to previously-conjugated ubiquitin molecules to form a polyubiquitin chain. If the chain is longer than 3 ubiquitin molecules, the tagged protein is rapidly degraded by the 26S-proteasome into small peptides (usually 3-24 amino acid residues in length). Al-Hakim (Al-Hakim et al., 2008) described a novel function for protein ubiquitination in regulating LKB1 signal transduction in a manner independent

of protein degradation. The ability of LKB1 to phosphorylate and activate some of the AMPK-related kinases is dependent upon their ubiquitin modification status. (Thomson et al., 1999). Ubiquitinated AMPK-related kinases are inactivated, deubiquitination of these proteins results in an release of inhibition, and subsequent LKB1 phosphorylation that would drive activation of the kinases. It remains to be seen if Jil-1 is ubiqutinated in Chriz RNAi animals and it would be interesting to see if normal Jil-1 activity is regulated by ubiquiniation.

4.4 Targeting of Jil-1 by Chriz/Z4 is required for the interbands

A strong reduction of the Jil-1 activity as a consequence of Chriz RNAi is strongly suggested by the diminished H3S10 phosphorylation observed on Western blots and in situ. Phosphorylation of H3S10 is related to two opposed chromatin states, namely the highly condensed mitotic chromosomes (driven by the Aurora kinase;(Hsu et al., 2000)) and the open chromatin of active genes and chromatin regions during interphase (reviewed by (Prigent and Dimitrov, 2003)). For instance, after heat shock phosphorylation of H3S10 accumulated in puff regions containing heat shock genes. However, phosphorylation targets not in all active promoters. (Labrador and Corces, 2003) Drosophila Jil-1 kinase localizes specifically to euchromatic interband regions of polytene chromosomes and it is the predominant kinase regulating histone H3S10 phosphorylation at interphase (Wang et al., 2001). Further analysis of Jil-1 demonstrated that reduction of Jil-1 results in the depletion of H3S10 phosphorylation and further result in chromosome perturbations. Recently (Deng et al., 2008) reported that targeted Jil-1 kinase results in locally decondensed chromatin. Using a LacI- tethering system, they showed that Jil-1 mediated ectopic histone H3S10 phosphorylation is sufficient to induce a change in higher-order chromatin structure within a condensed band to a more open state similar to interbands. That suggests that the normal Jil-1 function is to establish the decondensed state by phosphorylation. Once

the phosphorylation is reversed, then chromatin would be condensed again by the spreading of inactivating chromatin markers like H3K9me2 into such locations. This result fits with the observation of Rea (Rea et al., 2000) that H3S10 phosphorylation inhibits SUV39H1-dependent methylation of the adjacent H3K9 residue. The addition of negatively charged phosphate groups to histone tails also neutralizes their basic charge and is thought to reduce their affinity for DNA. Furthermore, it has been found that several acetyltransferases have increased HAT activity on serine 10-phosphorylated substrates, and that mutation of serine 10 decreases activation of Gcn5-regulated genes (Lo et al., 2000). Thus, phosphorylation may contribute to active chromatin through the inhibition of methylation and the stimulation of HAT activity on the same histone tail. Although the details still have to be elucidated I conclude that the Chriz/Z4 complex is required for targeting the Jil-1 kinase to interbands and for its stabilization to form and/or maintain locally open chromatin structure.

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Abbreviations

AA	Amino Acid
Ab	Antibody
AD	Activation Domain
Amp	Ampicillin
Bp	Base pair
BSA	Bovine serum Albumin
Chriz	Chromodomain protein interacting with Z4
CoIP	Coimmunoprecipitition
DBD	DNA Binding Domain
DEPC	Diethylpyrocarbonat
DNA	Deoxyribonucleic Acid
dNTP	Desoxynucleotidtriphosphat
E.coli	Escherichia coli
En	Engrailed
EDTA	Ethylendiamintertraacetat
FCS	Fetal Calf Serum
FL	Full Length
HAT	Histone Acetyl Transferase
IPTG	Isopropyl-B-D-thiogalactopyranosid
g	Gram
Gal.	Galatose
GST	Glutathion S-Transferase
Kb	kilobase
kDa	Kilodalton
LiAc	Lithiumacetat

Leu	Leucin
Lac	Lactose
M	Molar
Min	Minute
MH	Myc-His
mL	Milliliter
mM	Millimolar
NLS	Nuclear Localization signal
ONPG	O-Nitrophenyl-DGalactopyranosid
OD	Optical Density
PAGE	Polyacrylamid Gel Electrophoresis
PBS	Phosphate-Buggered-saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PMSF	Phenylmethylsulfonylfluorid
RNA	Ribonucleic Acid
RNAi	RNA interference
S. cerevisiae	Saccharomyces cerevisiae
SD	Synthetic Dropout
SDS	Sodium Dodocyl sulfate
Trp	Tryptophan
X-gal	5-brom-4-chlor-3-indolyl-B-D-galactopyranosid

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ERKLÄRUNG

Hiermit erkläre ich, Miao Gan, dass ich die vorliegenden Arbeit selbstständig verfasst, und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Berlin, den ....

Miao Gan